CFTRI-MYSORE
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Micro-analysis i.

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-is ⑨ hydrogen Ion concen
-tration ⑩ spectroscopic
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MICRO-ANALYSIS IN MEDICAL BIOCHEMISTRY

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CFTRI-MYSORE
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Micro-analysis I

Printed in Great Britain

PREFACE

DESCRIPTIONS of procedures for micro-analysis of certain commonly determined blood constituents have been given in a series of communications emanating from this laboratory during several years (King, *et al.*, 1936 to 1944). These methods have proved their usefulness in research and routine laboratory work and, since their publication, have met with a considerable measure of adoption in this country and abroad. This fact has encouraged research into new methods of blood analysis and to the introduction, where possible, of modifications which enable the determinations to be carried out on small quantities of material. Some of the procedures described in the original papers have since been modified, and micro-methods for other constituents have been investigated. The improvements introduced in the procedures formerly described are embodied in the descriptions given here, together with directions for the additional methods.

The trend of analytical research during recent years has been increasingly in the direction of micro-modification of existing procedures and in innovations of principle and technique which make it possible to perform the necessary manipulations with small amounts of material and reagents. The well-known work of Pregl and of Emich needs only to be mentioned as an illustration of this tendency. Biochemistry has not lagged behind other branches of the science in exploiting micro-analysis, and many authors have published the results of work which has been taken advantage of in developing the present set of procedures. Some methods have been adopted without change ; in others we have introduced our own modifications, refinements, and innovations. In all, we have kept rigidly to sound principles of chemistry and technique and have avoided the adoption of proposals which seemed to us to rest on doubtful chemical ground.

Micro-analysis enjoys an advantage which is perhaps particularly worth stressing at the present time. The manipulations are carried out with only a fraction of the material

which is usually used. When an analysis of blood is performed for, say, uric acid, on 0·2 ml. of blood instead of the 2 ml. which are usually used, the saving in reagents is very considerable. Many of the reagents for blood analysis are becoming increasingly difficult to obtain in any quantity, and any economy in their use is both a saving and a convenience, and may even be a necessity. The amount of labour necessary for making up reagents is also greatly reduced. Within our own experience, solutions last many times as long as they did with methods we used previous to adopting micro-procedures, for the simple reason that much less of them is used for analyses on the micro-scale.

The accuracy of the micro-methods is usually beyond question, and biochemical methods carried out on small quantities of capillary blood have given at least as consistent and as accurate results as the larger scale procedures from which they usually sprang. The advantage of being able to omit any coagulant substance in the taking of the specimen needs no comment. The sample can be measured in most capillary blood pipettes with a high degree of precision and the possibility of obtaining abnormal proportions of cells and plasma when sampling an improperly mixed specimen of venous blood (a potent source of error not often appreciated) is avoided.

The level of some substances in arterial blood is different from and of greater physiological significance than the level in venous blood. This is notably so in the case of glucose. Arterial blood glucose is best estimated in capillary blood which gives the same value.

Micro-chemical methods of blood analysis are particularly useful in investigations which require the taking of frequent samples of blood. Determinations on capillary blood as compared with venous blood are less laborious for the investigator and are less inconvenient to the patient, who usually objects to numerous and elaborate venipunctures. A puncturing apparatus or a Hagedorn needle, together with a supply of capillary blood pipettes, is much easier to keep and to use than a supply of sterilized syringes and needles. The micro-methods described for whole blood have been developed

primarily for use with capillary blood, but they are of course applicable to samples of venous blood.

Among the methods of blood analysis will be found certain procedures, i.e., cholesterol, calcium and CO_2-combining power, which have been adopted, unaltered, from the published descriptions of the original authors. Although we have introduced no modification or improvement in these methods, we have felt it advisable to include them for the sake of completeness, and in order to have together a set of instructions which make it possible to execute all the determinations which are commonly asked for in routine laboratory investigations. For the same reason, the tests and estimations on CSF and faeces are included, and those urine examinations which are essential for the simpler physiological tests of function.

The functional tests given are described in sufficient detail to enable them to be executed with precision, but no great space is devoted to discussing their clinical significance. Likewise no attempt is made to give a complete statement of the amounts of the various substances present in blood in diseased conditions nor to describe their significance at any length. Brief mention is made only of those clinical conditions in which abnormal values are most commonly encountered.

My former colleagues, Dr. G. A. D. Haslewood, Mr. G. E. Delory and Dr. D. Beall, have had an intimate share in the development of the procedures presented in this book. On their ideas and efforts I have largely depended for the elaboration of new analytical principles and the modification of existing ones to the needs of the micro-techniques. To them and to other colleagues, assistants and students, my thanks are due, for their counsel, collaboration, and loyal support.

E. J. K.

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MICRO-ANALYSIS IN MEDICAL BIOCHEMISTRY

Chapter I

NORMAL VALUES

By " normal value " is meant the amount of a constituent present in the body fluid or excretion of a healthy human being. In fact, this amount varies over a range, and while most healthy persons can be included in a class having the accepted " normal " amount, some individuals are found to show divergent figures. Such exceptional individuals are often entirely " normal " in all other investigated respects. The judgment, therefore, of whether a given analytical figure is " normal " will depend on the experience and total data at the command of the interpreter of the result.

The values given below are taken from the literature and from our accumulated results. In most cases they are assumed to apply to the method of analysis given here. This assumption is generally the result of a direct comparison between the method given and a method which has been " standardized for normal human beings " ; in other cases, inspection of a large collection of determinations prompts the feeling that the findings of a modified method do not differ significantly from those " accepted." It must be emphasized, however, that in several cases (uric acid in blood, for example,) no reliable data of the range of " normal value " exist ; it is hoped that when more general agreement as to methods to be used has been attained, such data will be forthcoming. Blood values are for fasting persons.

TABLE 1.—NORMAL VALUES

	per 100 ml.
WHOLE BLOOD	
Urea	20–40 mg.
Non-protein nitrogen	25–40 mg.
Uric Acid . . . . probably	2–4 mg.
"Creatinine"	1–2 mg.
Phosphate, inorganic (as P)—	
Adult	2–3 mg.
Child	4–5 mg.
Cholesterol	120–250 mg.
Sugar	60–100 mg.
Chloride (as NaCl)	450–510 mg.
PLASMA	
Total Protein	6·0–8·0 g.
Albumin	3·4–6·0 g.
Globulin	1·5–3·0 g.
(Ratio alb./glob. = 1·3–4·0)	
Fibrin	0·2–0·4 g.
Bilirubin	0·3–0·8 mg.
Chloride (as NaCl)	560–620 mg.
Sodium (as Na)	325–350 mg.
CO_2-combining power	55–75 ml.
Phosphate (as P)—	
ester	1 mg.
inorganic	2–3 mg.
lipide	4–7 mg.
Phosphatase, Acid	1–3 units
Alkaline	5–10 units
SERUM	
Calcium	9–11 mg.
Sodium	325–350 mg.
Potassium	16–20 mg.
CEREBRO-SPINAL FLUID	
Protein	20–40 mg.
Globulin (Pandy and Nonne Apelt Test)	absent
Chloride (as NaCl)	700–740 mg.
Sugar	60–100 mg.
Urea	15–30 mg.
Calcium	4–5 mg.
"Creatinine"	0·7–1·5 mg.
CO_2-combining power	55–65 vols.
FAECES	Percentage by weight of dried faeces.
A. Total fat	15–25
B. Unsoaped fat—	
(Neutral fat + Free fatty acid)	10–15
C. Free fatty acid	9–13
D. = A − B = Fatty acid present as soap	10–15
E. = B − C = Neutral fat	1–2

TABLE 2.—ABNORMALITIES IN COMPOSITION OF HUMAN BLOOD

Constituent	Clinical conditions in which high values (unless otherwise stated) are found
Plasma proteins (total)	Anhydremia. Low in nephritis with oedema (nephrosis), starvation.
Plasma albumin	Low in nephrosis.
Plasma globulin	Nephrosis, anaphylactic conditions.
CO_2-combining power	Alkalosis ($NaHCO_3$ administration, intestinal obstruction, over-breathing). Low in acidosis (diabetes, starvation, and severe nephritis).
Sugar	Diabetes, hyperthyroidism.
Non-protein N	Nephritis, eclampsia, intestinal obstruction, etc.
Urea	Nephritis, intestinal obstruction, etc.
Uric Acid	Nephritis, eclampsia, arthritis, gout.
Creatinine	Nephritis—only in severe cases above 4 mg./100 ml.
Chlorides (whole blood)	Nephritis, some cardiac conditions, eclampsia, prostatic obstruction, anaemia.
Chlorides (plasma)	Low in pneumonia, fever, diabetes; all cases of dehydration, such as gastro-intestinal disturbances associated with diarrhoea and vomiting.
Phosphates as P	Nephritis. Low in rickets.
Calcium (serum)	Hyperparathyroidism. Low in tetany (infantile), parathyroidectomy, severe nephritis, coeliac disease.
Cholesterol	Biliary obstruction, nephritis, nephrosis, diabetes, pregnancy. Low in pernicious anaemia.
Phosphatase	Generalized bone disease, obstructive jaundice.
Bilirubin	Jaundice.

Chapter II

PROCEDURES FOR WHOLE BLOOD

TAKING OF CAPILLARY BLOOD

Blood may be taken from a puncture in the ear or finger, but the most convenient place to obtain capillary blood is probably from the thumb over the bed of the nail. The part is wiped clean with a little ether or spirit and a stab of 1 to 2 mm. deep is made by means of a puncturing apparatus or Hagedorn needle. A piece of soft rubber tubing or of gauze is wrapped fairly tightly about the thumb above the knuckle. On flexing the thumb a free flow of blood is usually obtained. If the blood does not come easily, the rubber is released and the hand shaken in a downwards direction. This operation will ensure an adequate amount of blood when the tourniquet is replaced and the thumb flexed. The pipette is held horizontally with its point in the drop of blood issuing from the stab wound. The blood is allowed to run in exactly to the 0·2 ml. mark. The pipette is then wiped and the blood allowed to run into a 15 ml. conical centrifuge tube containing water or isotonic sodium sulphate solution, and by alternate blowing and sucking the pipette is washed several times with the solution.

TAKING OF VENOUS BLOOD

When several different estimations on whole blood are required it may be preferable to take a venous sample. Two ml. will usually suffice. The blood is withdrawn by a syringe from a vein in the antecubital fossa, according to the instructions given under *Plasma*, and is placed in a tube or screw-cap bottle containing a trace of potassium oxalate (the residue from a micro-drop of 30 per cent. dried in the tube).

UREA

Urea represents about 50 per cent. of the non-protein nitrogen of the blood. Normally there are between 20 and 40 mg. of urea present per 100 ml. High values are found in conditions associated with impaired renal function—particularly in chronic nephritis, but also in some cases of acute nephritis, prostatic obstruction, cardiac failure, etc.

PRINCIPLE

The sample of blood is digested with urease, and the urea thus converted into ammonia. After the removal of proteins, the colour produced by the ammonia with Nessler's reagent is compared colorimetrically with the colour produced under the same conditions with a standard ammonium chloride solution.

Direct Nesslerization does not lead to the production of cloudiness in the case of protein-free filtrates from unlaked blood. This is due to the fact that the sulphydryl substances, glutathione and ergothioniene, which produce turbidities with Nessler's reagent because of the insolubility of their mercury salts, are confined to the cells and do not appear in the filtrate, as is the case with filtrates of laked blood. Filtrates of unlaked blood have the further advantage that no ammonia is contributed to the determination through the action of the arginase of the red cells on the arginine contained in most commercial preparations of urease (see Addis, 1928). The use of zinc hydroxide as deproteinizing reagent eliminates a small amount of turbidity-producing substance contributed by most preparations of urease.

METHOD

0·2 ml. of blood is added to a centrifuge tube containing 3·2 ml. of isotonic sodium sulphate solution.

A " knife-point " (about 20 mg.) of Jack Bean meal is added, and the tube stoppered with a rubber bung, mixed, and incubated at 37° C. for 20 minutes. 0·3 ml. of zinc

sulphate solution and 0·3 ml. of 0·5 N-sodium hydroxide are added to precipitate the proteins. The mixture is well mixed by inversion after each addition and is then centrifuged. Two ml. of the supernatant fluid represent 0·1 ml. of blood.

Two ml. of the clear supernatant are treated with 5 ml. of ammonia-free distilled water and 1 ml. of Nessler's reagent. The solution is compared in a colorimeter with a " low " or " high " standard made up with 2 ml. or 5 ml. of the standard ammonium chloride solution (0·01 mg. of nitrogen per ml.), 5 ml. or 2 ml. respectively of water, and 1 ml. of Nessler's reagent*. The colorimetric comparison is facilitated by the use of a violet light filter.†

CALCULATION ‡

(1) " Low " standard :

$$\text{Blood urea}\ \S \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}02 \times \dfrac{100}{0{\cdot}1} \times 2{\cdot}14 \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 42{\cdot}8 \end{cases}$$

(2) " High " standard :

$$\text{Blood urea}\ \S \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}05 \times \dfrac{100}{0{\cdot}1} \times 2{\cdot}14 \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 107 \end{cases}$$

N.B.—1 mg. of nitrogen ≡ 2·14 mg. of urea

§ mg. per 100 ml. blood.

* If the blood urea is more than 150 mg. per 100 ml., the determination should be repeated with 1 ml. of supernatant (≡ 0·05 ml. blood).

† A Klett or Chance blue-violet light filter (obtainable from Messrs. Hearsons) is recommended for the colorimetric comparisons in the urea method, and for all comparisons of the Nessler yellow colour. This filter is particularly well suited for this comparison (see section on photometric measurement, p. 142). For photoelectric measurement of the colour a Chance blue-green or Ilford minus-red filter is suitable.

‡ The calculations given apply to results obtained with a Duboscq type colorimeter. For a photoelectric instrument certain modifications in the methods and in the form of the calculation are necessary. These are given on pp. 149, 150.

SOLUTIONS

Nessler's Reagent.—11·25 g. of iodine crystals are dissolved in a solution of 15 g. of potassium iodide in 10 ml. of water. 15 g. of mercury are added, and the mixture, kept cool in water, is shaken until the supernatant liquid has lost its yellow colour. This supernatant liquid is then decanted into a 100 ml. flask and a drop tested with 1 per cent. starch. If no colour is obtained, more iodine solution (prepared as above) is added until a drop of the mixture gives a faint reaction with starch.

The total solution is then diluted to 100 ml. and poured into 485 ml. of 10 per cent. sodium hydroxide. If the solution is turbid, it should be allowed to settle before use.

Standard Ammonium Chloride Solution (containing 0·01 mg. of nitrogen per ml.).—153 mg. of pure ammonium chloride are weighed out and dissolved in water. The volume is made to 100 ml.; 25 ml. of this solution with 10 ml. of N-sulphuric acid are diluted to 1 litre with distilled water.

Isotonic Sodium Sulphate.—Thirty g. of crystalline sodium sulphate ($Na_2SO_4.10H_2O$) are dissolved in water and made to 1 litre.

Zinc Sulphate.—Ten g. of crystalline zinc sulphate ($ZnSO_4.7H_2O$) are dissolved in water and made to 100 ml.

0·5 N-*Sodium Hydroxide.*—This should be accurately prepared, and should be titrated against the zinc sulphate. 10·8–11·2 ml. should be necessary to produce a permanent pink colour with phenolphthalein, when titrated into 10 ml. of zinc sulphate diluted with water.

NON-PROTEIN NITROGEN

The non-protein nitrogen containing substances of blood are urea (10–20 mg. N), uric acid (1–2 mg. N), creatinine (0·5–1 mg. N), amino-acid nitrogen (6–8 mg.), and substances such as glutathione and ergothioniene (5–10 mg. N per 100 ml. of blood). The normal range of non-protein (N.P.N.) is from 25–40 mg. per 100 ml. Increased values are found in the conditions showing a high blood urea.

PRINCIPLE

The proteins of laked blood or plasma are precipitated by tungstic acid. Part of the filtrate is digested with sulphuric acid until all the nitrogen is converted into ammonium sulphate. The ammonium salt is estimated colorimetrically with Nessler's solution, excess of which is used for the test in order to neutralize the sulphuric acid and give an alkaline medium.

METHOD

0·2 ml. of blood is laked with 3·2 ml. of water, or 0·2 ml. of blood in 3·2 ml. of isotonic sodium sulphate is laked by the addition of a drop of 1 per cent. saponin, followed by vigorous shaking. 0·3 ml. of 10 per cent. sodium tungstate and of $\frac{2}{3}$ N–H_2SO_4 are added and the mixture shaken and filtered or centrifuged.

One ml. of the filtrate (= 0·05 ml. of blood) is evaporated in a test-tube with 0·2 ml. of 50 per cent. sulphuric acid containing 1 per cent. SeO_2 until the liquid turns dark and white acid fumes are evolved. The addition of a small piece of porous pot prevents "bumping." Heating is continued until the mixture is colourless and for 3 or 4 minutes more. To the cooled solution are now added 5 ml. of water and, after thorough mixing, 3 ml. of Nessler's solution. The colour produced is compared in the colorimeter with the "low" or "high" standard used in the determination of blood urea.

CALCULATION

(1) "Low" standard :

$$\text{N.P.N.*} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}02 \times \dfrac{100}{0{\cdot}05} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 40 \end{cases}$$

* mg. per 100 ml. of blood or plasma.

(2) " High " standard :

$$\text{N.P.N.*} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}05 \times \dfrac{100}{0{\cdot}05} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 100 \end{cases}$$

* mg. per 100 ml. of blood or plasma.

SOLUTIONS

Nessler's reagent and *ammonium chloride* standard as described for urea.

Fifty per cent. Sulphuric Acid containing Selenium Oxide.— Fifty ml. concentrated acid are allowed to run slowly and with shaking into 50 ml. of distilled water in a 100 ml. volumetric flask. Selenium oxide (1 g. SeO_2) is added and shaken until dissolved. The mixture is cooled to room temperature, made to the mark, and mixed.

URIC ACID

Uric acid is normally present to the extent of 2–4 mg. per 100 ml. of blood. In gout and in certain conditions of renal impairment high values are found.

PRINCIPLE

Blood in isotonic sodium sulphate solution is treated with a phosphotungstic acid reagent. This precipitates the proteins, and on addition of sodium cyanide to the supernatant liquid, the excess of phosphotungstic acid reagent produces a blue colour with the uric acid present. The colour is compared with that given by a standard solution of uric acid. The blood must not be laked, as interfering substances, such as glutathione and ergothioniene, would be liberated from the cells.

METHOD

0·2 ml. of capillary blood is pipetted into 3·2 ml. of isotonic sodium sulphate in a 15 ml. centrifuge tube. 0·6 ml. of Folin's (1934) uric acid reagent is added. The tube is

stoppered, its contents mixed gently by inversion, and immediately centrifuged. Two ml. of the supernatant liquid (= 0·1 ml. of blood) are treated with 1 ml. of sodium cyanide-urea reagent. At the same time a mixture of 1 ml. of the uric acid " blood " standard (= 0·004 mg. uric acid), 0·7 ml. of distilled water, and 0·3 ml. of Folin's reagent is also treated with 1 ml. of sodium cyanide-urea reagent. The two tubes are placed in a boiling water-bath for 5 minutes, cooled, and the solutions compared colorimetrically * (red or orange light filter).

CALCULATION

$$\text{Uric acid } \dagger \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}004 \times \dfrac{100}{0{\cdot}1} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 4 \end{cases}$$

† mg. per 100 ml. blood.

SOLUTIONS

Isotonic Sodium Sulphate as under blood urea.

Sodium Cyanide-urea Reagent.—Five g. of sodium cyanide and 20 g. of urea are dissolved in water, and the volume made to 100 ml. The urea prevents clouding during the determination. This solution should be renewed about once a month.

Folin's (1934) Uric Acid Reagent.

1. Preparation of molybdate-free sodium tungstate: a solution of 250 g. of sodium tungstate in 500 ml. of water is treated with 5 N-hydrochloric acid until neutral to litmus paper. The solution is saturated with hydrogen sulphide, and allowed to stand 24 hours. It is then treated with 400 ml. of absolute alcohol, added gradually with constant shaking. The mixture, after standing for a further 24 hours, is filtered, and the precipitate washed with 50 per cent. alcohol and dissolved in 375 ml. of water. 0·5 ml. of bromine is added, and the mixture boiled gently until the excess bromine is dispelled.

* Micro-cups for an ordinary Duboscq colorimeter are necessary for this and certain other procedures, e.g., creatinine, bilirubin.

Sodium hydroxide solution (40 g. per 100 ml.) is now added to the hot solution until the latter is alkaline to phenolphthalein. The cooled solution, filtered if necessary, is treated with 200 ml. of absolute alcohol, and allowed to stand for 24 hours. The white crystals are filtered off and dried in a desiccator.

2. Preparation of reagent: 100 g. of molybdate-free sodium tungstate are treated gradually with a solution of 30 ml. of " syrupy " phosphoric acid (89 per cent.) in 150 ml. of water. The mixture is boiled gently under reflux for 1 hour, decolourized as above with a drop of bromine, cooled and diluted to 500 ml.

Stock Uric Acid Standard (*Folin*) (= 1 mg. per ml.).—One g. of uric acid is placed in a 1-litre flask. 0·6 g. of lithium carbonate is dissolved in 150 ml. of cold water. The carbonate solution, filtered if necessary, and warmed to 60° C., is added to the flask containing the uric acid, which is warmed under the hot tap. The warm mixture is shaken for five minutes, cooled at once under the tap, and treated with 20 ml. of formalin (40 per cent. solution of formaldehyde) and enough water to fill half the flask. A few drops of methyl orange are added, and then, gradually with shaking, 25 ml. of N-sulphuric acid. The solution should turn pink when 2–3 ml. of acid remain to be added. The mixture is now diluted to 1 litre, mixed and stored in the dark in a stoppered bottle, when it will keep almost indefinitely.

Uric Acid " Blood " Standard (= 0·004 mg. per ml.).—Two ml. of the above " stock " standard solution are diluted with water and 1 ml. of 40 per cent. formalin to 500 ml. This solution should be made up fortnightly.

CREATININE

Colorimetric Estimation

PRINCIPLE

Creatinine gives a red colour with alkaline solutions of picric acid (Jaffe's reaction). A similar colour is also given by blood (and plasma) filtrates. It is not certain that the

colour in this case is due to creatinine, but the substance which may thus be estimated as blood " creatinine " is of some clinical importance. Calculated as " creatinine " the normal values are 1–2 mg. per 100 ml. of blood. In advanced renal failure enhanced values may be found.

METHOD

0·2 ml. of blood is added to 1·4 ml. of isotonic sodium sulphate solution. 0·2 ml. of 10 per cent. sodium tungstate and 0·2 ml. of $\frac{2}{3}$ N-sulphuric acid are added, and the tube is stoppered and shaken. The mixture is centrifuged, and 1 ml. of the clear supernatant fluid (= 0·1 ml. of blood) is used as " test " solution (see Somogyi, 1930).

For normal blood a " standard " solution is made by diluting 1 ml. of the creatinine " blood " standard described below, with 4 ml. of water. The " test " solution and 1 ml. (= 0·001 mg. of creatinine) of this " standard " solution are then treated at the same time with 0·5 ml. of freshly made alkaline picrate solution (see below). After not more than 15 minutes the solutions are compared in the colorimeter, using a blue-green light filter, such as Ilford's spectrum blue-green.

CALCULATION

$$\text{Blood "creatinine"}^{*} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}001 \times \dfrac{100}{0{\cdot}1} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 1{\cdot}0 \end{cases}$$

* mg. per 100 ml. blood.

In cases where a raised blood " creatinine " is found or expected, stronger " standards " may be made by using larger quantities of the " blood standard," and diluting these as before to 5 ml. with water. In general the calculation becomes :—

$$\text{Blood "creatinine"} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times \text{C} \times 0{\cdot}001 \times \frac{100}{0{\cdot}1}$$

where C is the number of ml. of " blood " standard used in the 5 ml. of " standard " solution.

CREATININE

Photometric Estimation

The amount of creatinine in blood is so small that the amount of colour in a test, which is contributed by the excess of reagent (alkaline picrate solution), is appreciable compared with that produced by the reaction of creatinine with reagent. The difficulty of obtaining an accurate assessment of the colour due to creatinine can be overcome by using the colorimeter as a photometer in the manner described by Delory and Jacklin (1942).

The neutral grey screen (density 0·50; cf., p. 142) is placed on the left-hand rack of the colorimeter, and the Ilford blue-green filter placed over the eyepiece. The standard solution is placed in the right-hand cup and its depth is adjusted until the two fields appear equal. The reading S is recorded. The test solution is now substituted in the cup to give the reading T. Finally, a blank consisting of 1 ml. of water and 0·5 ml. of alkaline picrate solution is read (reading B).

CALCULATION

$$\text{Blood "creatinine"}^{*} \begin{cases} = \dfrac{1/T - 1/B}{1/S - 1/B} \times 0{\cdot}001 \times \dfrac{100}{0{\cdot}1} \\ = \dfrac{\dfrac{B - T}{BT}}{\dfrac{B - S}{BS}} \times 1 \\ = \dfrac{S}{T} \times \dfrac{B - T}{B - S} \times 1 \end{cases}$$

* mg. per 100 ml. of blood.

If it is desired to avoid this calculation, a curve is made from the readings obtained with varying strengths of standard. Results can then be read off from this curve. Since different preparations of picric acid may contain varying amounts of impurities (e.g., dinitrophenol), it is advisable to check the curve for each new batch of picric acid.

SOLUTIONS

Isotonic Sodium Sulphate, ten per cent. zinc sulphate and 0·5 N-*sodium hydroxide* as under blood urea.

Creatinine "Stock" Standard (containing 1 mg. of creatinine per ml.).—1·602 g. of pure creatinine zinc chloride are dissolved in N/10 hydrochloric acid solution and the volume made with the N/10 acid to 1 litre.

Creatinine "Blood" Standard (0·005 mg. per ml.).—Five ml. of the above "stock" standard are treated with 10 ml. of N/10 hydrochloric acid and the volume made with water to 1 litre.

Alkaline Picrate Solution.—Five parts (by volume) of a saturated aqueous solution of *pure* picric acid, containing about 15 g. picric acid per litre, are mixed with 1 part (by volume) of 10 per cent. sodium hydroxide.

NOTE.—The picric acid may be purified by recrystallization from glacial acetic acid. It must be of such purity that when 10 ml. of a saturated aqueous solution are treated with 5 ml. of the 10 per cent. hydroxide, the colour (determined in the colorimeter) of the alkaline mixture so formed is not more than twice as deep as that of the saturated picric acid solution. The alkaline picrate must be prepared freshly for use.

INORGANIC PHOSPHATE

(Adapted from the method of King, 1932)

The blood of normal adult persons contains 2–3 mg. per 100 ml. (expressed as P) of inorganic phosphate. In conditions involving an acidosis, such as is often found in nephritis, the amount present may be definitely raised. The amount of phosphate present in the blood of children, where bone formation is not yet complete, is at a higher level—usually of about 5 mg. per 100 ml. In rachitic conditions the figure is lowered.

PRINCIPLE

The inorganic phosphate of a deproteinized filtrate of the blood is coupled with molybdate, and the yellow phospho-

molybdate is reduced to give a blue substance. The amount of blue colour produced in the solution is directly proportional to the amount of phosphate present.

METHOD

0·2 ml. of whole blood or plasma is pipetted into 3·2 ml. of water or isotonic sodium sulphate and treated with 0·6 ml. of 25 per cent. trichloroacetic acid. The mixture is shaken well, and after 5 minutes filtered through a small paper. 2 ml. of the clear filtrate (= 0·1 ml. of blood or plasma) are treated at the same time as 2 ml. of the dilute standard phosphate solution (= 0·004 mg. P) with 0·3 ml. of the ammonium molybdate solution followed by 0·2 ml. of the reducing agent (aminonaphtholsulphonic acid). The contents of the tubes are gently shaken between each addition and the colours are read in a colorimeter after 10 minutes (red or orange light filter).

CALCULATION

$$\text{Blood phosphate}^{*} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}004 \times \dfrac{100}{0{\cdot}1} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 4 \end{cases}$$

* mg. per 100 ml. of blood.

SOLUTIONS

Trichloroacetic Acid Solution.—Twenty-five g. of pure trichloroacetic acid dissolved in water, and diluted to 100 ml. and mixed.

Acid Ammonium Molybdate.—Five g. ammonium molybdate are added to a mixture of 75 ml. distilled water and 15 ml. of concentrated sulphuric acid in a 100 ml. volumetric flask. The mixture is shaken until dissolution is complete and cooled to room temperature. The solution is then made up to 100 ml. and mixed.

Reducing Agent. 0·2 per cent. aminonaphtholsulphonic

acid.—0·2 g. of the 1 : 2 : 4-acid, 12 g. sodium meta-bisulphite and 2·4 g. crystalline sodium sulphite are dissolved by shaking with enough water to make 100 ml. If the solution does not filter clear it should be left overnight and filtered again. A fresh solution should be prepared every two weeks.*

Standard Phosphate.—A stock solution is made by dissolving 2·194 g. of pure potassium dihydrogen phosphate (KH_2PO_4) in 500 ml. in water. This solution contains 1 mg. P per ml. A *dilute standard solution* is made by diluting 2 ml. of the stock solution to 1 litre with water. One ml. of this solution contains 0·002 mg. P. Both solutions should be kept saturated with chloroform to prevent any bacterial growth, which might cause a loss of inorganic phosphate.

CHOLESTEROL

(Method of Sackett, 1925)

Total Cholesterol

Total cholesterol in the blood of normal persons may be present in amounts varying from 120 to 250 mg. per 100 ml. The amounts present in blood may be raised in severe diabetes, biliary obstruction, and in some forms of nephritis (particularly the nephrotic type).

PRINCIPLE

Blood is treated with an alcohol-ether mixture, which extracts the cholesterol and at the same time precipitates the proteins. The extract is evaporated to dryness, and the cholesterol in it determined by means of the Liebermann-Burchard reaction. This comprises the production of a green colour when chloroform solutions of certain sterols are treated with acetic anhydride and sulphuric acid.

* The reducing agent may be prepared fresh for use from a tablet containing the correct amounts of 1 : 2 : 4–aminonaphtholsulphonic acid, sodium sulphite and sodium meta-bisulphite. A tablet is ground with 10 ml. of water, filtered, and the solution is ready for use (Messrs. Gallenkamp).

METHOD

0·2 ml. of blood is pipetted drop by drop into a centrifuge tube containing a mixture of 8 ml. of absolute alcohol and 2 ml. of ether. The stoppered tube is shaken vigorously for about 1 minute, and is then allowed to lie horizontally, with an even distribution of the precipitate along the tube, for 30 minutes. The mixture is then centrifuged, and the supernatant liquid poured, as completely as possible, into a small beaker. This is placed on a water-bath or hot plate, and the contents carefully evaporated to dryness. The residue is washed out with about 4 ml. of chloroform, in three portions, into a glass-stoppered 10 ml. measuring cylinder, and the volume made to 5 ml. with chloroform.

0·5 ml. (≡ 0·5 mg. of cholesterol) of the cholesterol standard is added to a similar container and diluted to 5 ml. with chloroform. To each solution are added 2 ml. of acetic anhydride, and 0·1 ml. of concentrated sulphuric acid. The cylinders are stoppered, and their contents mixed and allowed to stand in the dark for 10 minutes. The two solutions are then compared in the colorimeter. (Red or orange light filter.)

CALCULATION

$$\text{Total cholesterol}^{*} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}5 \times \dfrac{100}{0{\cdot}2} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 250 \end{cases}$$

* mg. per 100 ml. of blood.

Cholesterol Standard.—0·1 g. of pure cholesterol in 100 ml. of chloroform.

GLUCOSE

Titrimetric method for "True Sugar"

Harding's (1932, 1933) modification of the Schaffer-Hartmann method. This method gives "true sugar values" as opposed to total reducing substances when applied to the filtrate of unlaked blood described below.

PRINCIPLE

The " sugar " in whole blood is a mixture of glucose, present mainly in the plasma, and nitrogenous reducing substances (chiefly glutathione) in the corpuscles. It is possible to exclude the corpuscles by mixing the blood with isotonic sodium sulphate solution, in which the corpuscles remain intact. A determination of the reducing power then becomes equivalent to an estimation of glucose alone. For normal (fasting) individuals values of 65–90 mg. per 100 ml. are found (King *et al.* 1937).

A protein-free filtrate of blood, and alkaline copper reagent are heated together. Part of the copper is thereby reduced and is equivalent to the amount of reducing substances (sugar) present. The degree of reduction is determined indirectly by reoxidizing the reduced copper with iodine and determining the amount of iodine consumed in the process. The reagent contains potassium iodate, from which iodine is liberated by the addition of potassium iodide and sulphuric acid. The iodine set free both in the test and in a " blank " determination (2 ml. of copper reagent and 2 ml. of water), is determined by titration with sodium thiosulphate. The difference in the two titrations represents the amount of iodine consumed in reoxidizing the reduced copper.

METHOD

The blood is taken into a dilute copper solution, in which there is no loss of sugar by glucolysis (King, Pillai and Beall, 1941). 0·2 ml. of capillary blood is washed from a pipette into 3·5 ml. of isotonic sodium sulphate-copper sulphate solution. One of the Somogyi (1931) protein precipitating reagents is thus used for the preservation of the blood. When it is desired to complete the analysis, 0·3 ml. of 10 per cent. sodium tungstate is added ; the copper tungstate formed precipitates the protein. The mixture is shaken and then centrifuged.

Two ml. of the supernatant liquid (= 0·1 ml. of blood) are treated with 2 ml. of the mixed copper reagent in a wide

($\frac{3}{4}$ inch) test-tube. A " blank " is prepared with 2 ml. of distilled water and 2 ml. of reagent. Both tubes, stoppered lightly with cotton wool, are placed in a boiling water bath for exactly 10 minutes. They are then cooled at once under the tap. To each is added 1 ml. of 2 per cent. potassium iodide and 1 ml. of 2 N-sulphuric acid. After standing 1 minute the contents of each tube are titrated with N/200 sodium thiosulphate. One per cent. soluble starch (made up in water or, better, in saturated phenol red solution) is used as indicator. The titration figure of the test solution is subtracted from that of the " blank."

CALCULATION

1 ml. N/200 thiosulphate = 0·116 mg. glucose

The ml. of thiosulphate given by the difference between the " blank " and " test " titrations is equivalent to the amount of glucose present in the " test." Hence :—

ml. N/200 thiosulphate × 0·116 = mg. glucose in 2 ml. filtrate (i.e., in 0·1 ml. blood)

And therefore :—

$$\left.\begin{array}{l}\text{ml. N/200 thiosulphate} \\ \times\ 0{\cdot}116 \times \dfrac{100}{0{\cdot}1}\end{array}\right\} = \text{mg. of glucose per 100 ml. blood}$$

i.e.,

$$\left.\begin{array}{l}\text{ml. N/200 thiosulphate} \\ \times\ 116\end{array}\right\} = \text{mg. of glucose per 100 ml. blood.}$$

If the blood-sugar value thus obtained is greater than 400 mg./100 ml., the determination should be repeated, using as test solution a mixture of 1 ml. of filtrate and 1 ml. of water. The result then obtained is multiplied by 2.

SOLUTIONS

Isotonic Sodium Sulphate-Copper Sulphate Solution (to prevent glucolysis).—A mixture of 320 ml. of 3 per cent. sodium sulphate and 30 ml. of 7 per cent. copper sulphate ($CuSO_4.5H_2O$).

Sodium Tungstate.—10 g. per 100 ml.

Copper Reagent (Harding's modification of the Schaffer-Hartmann reagent).

Solution A consists of :—

13 g. Copper sulphate crystals dissolved in water and the volume made to 1 litre.

Solution B is made by dissolving :—

24 g. Rochelle salt (sodium potassium tartrate)
40 g. Anhydrous sodium carbonate
50 g. Sodium bicarbonate
36·8 g. Potassium oxalate, and exactly
1·4 g. Potassium iodate.

Solution B is best prepared as follows :—

The bicarbonate is weighed out, washed into a litre flask, and dissolved at room temperature in about 700 ml. of water. The carbonate is now washed in, and dissolved, also without warming. The oxalate is weighed into a beaker and washed with successive quantities of warm water, the washings being added to the main solution, until all the oxalate is dissolved. The Rochelle salt is dissolved in a little water in a beaker, and washed into the mixture ; finally, the iodate is weighed out and washed directly into the solution which is well shaken, made to the mark, and again thoroughly mixed.

The " copper reagent " used in the above method is a freshly made mixture of exactly equal volumes of solutions A and B.

NOTE.—Only purest analytical chemicals should be used in making up the above reagent. When preparing a fresh copper reagent it is advisable to check it against a solution of pure glucose.

GLUCOSE

Colorimetric Method for " True Sugar " in 0·05 ml. of Blood

PRINCIPLE

With this method (King and Garner, 1947) glucose is estimated accurately in pure solution and in blood. The

results obtained are identical with those found with the previously described titration method. The proteins are precipitated by sodium tungstate and copper sulphate (Somogyi, 1931) and the filtrate is treated with a modified Harding and Downs (1933) copper reagent, from which the iodate is omitted. The cuprous oxide formed is estimated by the colour produced with an arseno-molybdic acid solution (Nelson, 1944).

METHOD

0·05 ml. of whole blood is pipetted into 1·85 ml. of isotonic sodium sulphate-copper sulphate solution in a conical centrifuge tube. 0·1 ml. of sodium tungstate is added, and the mixture is well shaken. The precipitated proteins and copper tungstate are spun down in the centrifuge. 1 ml. of the supernatant fluid (≡ 0·025 ml. of blood) is mixed with 1 ml. of the mixed copper reagent in a $\frac{3}{4}$ in. diameter test-tube. The tube, stoppered with cotton wool, is placed in a boiling water-bath for exactly 10 minutes. After immediate cooling, 3 ml. of the arseno-molybdic acid reagent are added. The colour is compared, after 10 minutes, with that produced by 1 ml. of a standard glucose solution in benzoic acid, treated in the same way as the blood filtrate. (Red or orange filter.)

CALCULATION

Let X = concentration of standard in mg. per ml.

$$\text{Blood sugar } * = \frac{\text{Reading of standard}}{\text{Reading of test}} \times X \times \frac{100}{0{\cdot}025}$$

e.g., with 0·02 mg. per ml. standard, Blood sugar *

$$= \frac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}02 \times \frac{100}{0{\cdot}025}$$

$$= \frac{\text{Reading of standard}}{\text{Reading of test}} \times 80$$

Similarly, with 0·05 mg. per ml. standard, Blood sugar *

$$= \frac{\text{Reading of standard}}{\text{Reading of test}} \times 200$$

* mg. per 100 ml. blood.

SOLUTIONS

Solution A.—Thirteen g. $CuSO_4.5H_2O$ are dissolved in water and the volume made to 1 litre.

Modified Solution B.—Fifty g. $NaHCO_3$ are dissolved in a beaker with stirring in the minimum amount of distilled water (about 700 ml.). When all the bicarbonate is dissolved, 40 g. anhydrous Na_2CO_3 are added with stirring to the solution. When the carbonate has dissolved, a solution of 36·8 g. potassium oxalate in 120 ml. of warm water is added to the mixture. Finally, a solution of 24 g. sodium potassium tartrate in the minimum amount of water (about 100 ml.) is added. The mixture is poured and washed into a 1 litre volumetric flask, the volume is made to the mark, and the solution well shaken.

The *copper reagent* used is prepared freshly each day and is a mixture of exactly equal volumes of solutions A and B.

Arseno-molybdic Acid Reagent (Nelson, 1946).—Twenty-five g. ammonium molybdate are dissolved in 450 ml. of water; 21 ml. conc. sulphuric acid added, mixed, and then 3 g. of sodium arsenate ($Na_2HA_sO_4.7H_2O$) dissolved in 25 ml. of water. The mixture is kept in the 37° bath for 2 days, and is preserved in a brown bottle. One volume of this reagent is diluted with 2 volumes of water for use.

Isotonic Sodium Sulphate-Copper Sulphate Solution.—A mixture of 320 ml. of 3 per cent. sodium sulphate ($Na_2SO_4.10H_2O$) and 30 ml. of 7 per cent. $CuSO_4.5H_2O$.

Sodium Tungstate.—Ten g. per 100 ml.

Stock Glucose Solution.—0·1 g. pure anhydrous glucose is dissolved in saturated benzoic acid solution (0·3 per cent.) and the volume made to 100 ml. This is a permanent standard.

Standard Glucose Solutions.—These are prepared by diluting 2, 3, and 5 ml. of the stock glucose solution to 100 ml. with saturated benzoic acid solution; giving standards of 0·02, 0·03 and 0·05 mg. per ml. (equivalent to 80, 120 and 200 mg. glucose per 100 ml. of blood).

GALACTOSE

Galactose is not a normal constituent of the blood. It is present in considerable amount after oral or intravenous

administration, and measurement of the rate at which it disappears from the blood is used as a test of liver function (King and Aitken, 1940), and of thyrotoxicosis (Barnes and King, 1942).

PRINCIPLE

The glucose can be completely removed from diluted blood by fermentation with washed baker's yeast. Galactose remains unattacked and can be estimated in the deproteinized filtrate of the blood by the copper reduction method used in the estimation of blood glucose.

METHOD

Fresh baker's yeast (2 g.) is well washed by shaking with distilled water (10 ml.) in a centrifuge tube. The yeast is spun down ; stirred up with a fresh 10 ml. of water, well shaken, and again centrifuged. The washing is repeated a third time. The yeast is finally stirred and shaken with 10 ml. of isotonic sodium sulphate and is ready for use.

The blood sample (0·2 ml.) is added to 2·5 ml. of a mixture of 22 ml. of isotonic sodium sulphate and 3 ml. of 10 per cent. sodium tungstate in a centrifuge tube. One ml. of yeast suspension is added. The contents of the tube are thoroughly mixed with the aid of a glass rod, and the tube is then incubated in a water thermostat at 37° C. for 15 minutes.

The proteins are precipitated by the addition of 0·3 ml. of 7 per cent. copper sulphate. The proteins and the yeast are filtered off or centrifuged (for 5 min.) and the galactose determined in 2 ml. of the supernatant fluid (equivalent to 0·1 ml. of blood) by the titration method described for blood glucose.

A blank determination consisting of 2·7 ml. of isotonic sulphate-tungstate and 1 ml. of yeast suspension is carried through at the same time as the other tests. The blank determination on the yeast should give the same figure in ml. of thiosulphate as is given by the blank determination with water, which is always carried out in the sugar

determination. The yeast should also be tested against pure glucose and against galactose. 0·2 ml. of a 100 mg. per 100 ml. galactose solution is analysed with and without the addition of yeast. The same reducing power should be found in both filtrates. (No preservative should be used in preparing these sugar solutions.)

CALCULATION

The factor 162, multiplied by the difference between the ml. of thiosulphate used in test and blank, gives the mg. of galactose per 100 ml. of blood.

SULPHONAMIDES

Sulphanilamide appears in the blood in variable amount after administration. Part of it is present in the free state and part in a conjugated form. For therapeutic purposes it is usual to try to adjust the amount administered by mouth so as to maintain a level of 8–15 mg. of the free sulphanilamide per 100 ml. of blood. The total sulphanilamide (i.e., free plus conjugated) is usually 20–50 per cent. more than the free.

PRINCIPLE

The blood is deproteinized with trichloroacetic acid. Sulphanilamide is estimated in the filtrate by means of the diazo reaction. The sulphanilamide is diazotized with sodium nitrite, the excess nitrite is destroyed by ammonium sulphamate, and the diazonium compound is coupled with naphthyl ethylene diamine. The pink colour produced is compared with that developed from a standard sulphanilamide solution treated in the same way. The conjugated sulphanilamide must be released from its organic combination before it will react with the reagents. This is accomplished by boiling the trichloroacetic acid filtrate with hydrochloric acid. Determination of the sulphanilamide in the resultant solution gives the total sulphanilamide, free plus conjugated.

The chemically related drugs, sulphapyridine and sulphathiazole, are determined in the same way.

METHOD

This method is adapted from the procedure of Bratton and Marshall (1939). 0·2 ml. of blood is added to 3·2 ml. of water or isotonic sodium sulphate; 0·6 ml. of 25 per cent. trichloroacetic acid is added. The mixture is vigorously shaken, and filtered or centrifuged; 2 ml. of the filtrate (≡ 0·1 ml. of blood) are transferred to a test-tube and 1 drop of sodium nitrite solution added. The tube is shaken and left for 3 min.; 1 ml. of ammonium sulphamate solution is added and the mixture left for 2 min. with occasional shaking. Two ml. of naphthyl ethylene diamine solution are now added and the mixture shaken. The coloured solution is compared with a standard prepared in the same way from 2 ml. of standard solution (≡ 0·004 mg. sulphanilamide). The use of a yellow-green light filter (e.g., Ilford spectral yellow-green) facilitates the comparison.

Total sulphanilamide is determined by heating 2 ml. of filtrate with 0·5 ml. of N-hydrochloric acid in a 5 ml. volumetric flask in a boiling-water bath for 1 hour. The cooled contents of the flask are then treated with sodium nitrite, &c. as in the procedure for free sulphanilamide, and the volume adjusted to 5 ml. with water.

CALCULATION

$$\text{Blood sulphanilamide } * \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}004 \times \dfrac{100}{0{\cdot}1} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 4 \end{cases}$$

* mg. per 100 ml. blood.

If the colour of the test is more than twice as strong as that of the standard, the determination should be repeated with 1 ml. of filtrate plus 1 ml. of water.

If sulphapyridine or sulphathiazole is determined by the above procedure, and with a sulphanilamide standard, the value obtained is multiplied by 1·4 to give the mg. sulpha-

pyridine or sulphathiazole per 100 ml. blood. Factors for other sulphonamides are calculated from the ratios of their molecular weights to that of sulphanilamide.

SOLUTIONS

Twenty-five per cent. Trichloroacetic Acid.—Twenty-five g. of the acid dissolved in water and made to 100 ml.

Sodium Nitrite Solution.—0·5 g. dissolved in 100 ml. of water. This solution is the same as solution B in the bilirubin method.

Ammonium Sulphamate Solution.—0·5 g. dissolved in water and made to 100 ml.

Naphthyl Ethylene Diamine Solution.—0·05 g. of N-(1-naphthyl) ethylene diamine dihydrochloride in 100 ml. of water ; stored in a brown bottle.

Stock Standard Sulphanilamide (0·1 mg. per ml.).—100 mg. of sulphanilamide are dissolved in 1 litre of water.

Standard Solution (0·002 mg. sulphanilamide per ml.).—2 ml. of the stock standard, together with 15 ml. of the 25 per cent. trichloroacetic acid, are diluted to 100 ml. with water.

HAEMOGLOBIN

Haemoglobinometry has two chief functions—first, the detection of diseases characterized by deficiency or excess of haemoglobin ; secondly, the study of changes in haemoglobin concentration caused by loss or gain of plasma. For both purposes it is desirable that the method employed should estimate the concentration of all the forms of haem-pigment circulating in the blood. Ammundsen (1939, 1941) has shown that under modern conditions a normal adult may have from 2 to 12 per cent. of his total haemoglobin circulating in an inactive form. The inactive fraction is chiefly composed of carboxyhaemoglobin, methaemoglobin and sulphaemoglobin. Drugs of the sulphonamide class often increase the proportion of inactive haemoglobin in patients receiving this form of treatment. Most methods of estimating haemoglobin are standardized by reference to determinations of

the oxygen-carrying power of blood. These yield valuable information concerning one important function of the blood, but they may provide a false picture of the pigment metabolism, since they take no account of inactive haemoglobin which may be capable of regeneration to the active form. They may imply the presence of anaemia where none exists. For this reason it has been deemed preferable (Clegg and King, 1942) to utilize the iron content of blood as a measure of its haemoglobin content. Blood contains about 50 mg. of iron per 100 ml. and almost all of this is present in the form of haemoglobin. Only a fraction of a mg. of non-haemoglobin iron is present in normal blood and this is largely confined to the plasma. Since all forms of haemoglobin contain iron in the same proportion, its estimation serves to give the total haemoglobin content of the blood.

Authorities differ as to the percentage of haemoglobin in blood which they regard as a normal figure. The three most commonly used are given in Table 3. There is an increasing body of evidence to show that Haldane's (1900) figure of 13.8 g. of haemoglobin is too low and that the Haden (1922) value of 15·6 g. per 100 ml. (20·9 ml. O_2) is a more normal figure (cf., Peters and Van Slyke, 1932; Wardlaw, 1941). This is the figure we have used and which has seemed adequately to represent our normal healthy cases; it has given colour indices averaging unity for healthy persons and treated anaemias.

TABLE 3.—*Concentrations of "Normal" Blood Standards*

	Haldane	Haden	Sahli
g. hæmoglobin per 100 ml. . .	13·8	15·6	17·2
O_2-capacity (ml. per 100 ml.) . .	18·5	20·9	23·0
mg. Fe per 100 ml.	46·2	52·2	57·6

Four methods for estimating haemoglobin are given. The alkaline and the cyan-haematin methods estimate the total haemoglobin. The cyan-methaemoglobin method estimates oxidized and reduced haemoglobin, carboxyhaemoglobin and

methaemoglobin. And the carboxyhaemoglobin method estimates oxidized, reduced and carboxyhaemoglobin.

PRINCIPLES OF METHODS

Alkaline Haematin. The haem — the pigmented constituent of the haemoglobin molecule—is split off from the protein part (globin) by treatment with alkali. The process may be accelerated by heat or by a preliminary treatment with acid. The brown solution of alkaline haematin, so produced, is compared with a standard solution of crystalline haemin in sodium hydroxide.

Cyan-methaemoglobin. The haemoglobin solution is treated with ferricyanide which turns most forms of haemoglobin into the pale yellow methaemoglobin. The cyan derivative of methaemoglobin is intensely red-coloured.

Cyan Haematin. Haemoglobin is treated with HCl and then with NaCN, and the red colour compared with a standard solution of crystalline haemin in cyanide.

Carboxyhaemoglobin. The solution of haemoglobin is treated with carbon monoxide from a cylinder or generator, or with coal gas which contains carbon monoxide. The cherry-red colour of the carboxyhaemoglobin, so produced, is matched colorimetrically against a standard solution, or photometrically with a green light filter.

METHODS

Standards of Colorimetric Reference

Photometric. The most convenient standards are Ilford grey screens. These serve as photometric standards for any coloured solution read with a suitable light filter (cf., King, Gilchrist and Delory, 1944). They are inexpensive, easily obtainable and are apparently permanent if kept away from heat and from light when not in use. By comparing a grey screen against a few bloods of known haemoglobin content (by iron or O_2 analyses) with an Ilford mercury green light filter over the eye-piece of a Duboscq colorimeter, or a Chance green filter in a photoelectric instrument, it is possible to assign a haemoglobin value to the grey screen, which may

thereafter be used as a standard. Grey screens are mounted in optical glass and are procurable in any size and shape to fit any instrument.

Colorimetric. The haemin standard recommended by Clegg and King (1942) may be used interchangeably with a neutral grey screen for the comparisons in the alkaline haematin method. It is convenient to standardize a grey screen in combination with an Ilford mercury green filter in a Duboscq, or with a Chance green filter in a photoelectric colorimeter, by reference to a standard haemin solution. The haemin used by us for all standard solutions is British Drug Houses' crystalline haemin. 79·4 mg. of pure haemin (8·57 per cent. Fe) dissolved in 1 litre of N/10 NaOH gives the same intensity of colour as blood of 15·6 g. haemoglobin per 100 ml. when diluted 1 in 100 in soda, and compared, using a green light filter. Samples of haemin of other than theoretical iron content should be used proportionately, e.g. 81 mg. of B.D.H., 8·41 per cent. Fe haemin ($79{\cdot}4 \times 8{\cdot}57/8{\cdot}41 = 81$). New standards should be prepared at regular intervals—e.g. of two or three months.

For the cyan-haematin method a standard solution of 30·4 mg. of pure haemin of 8·57 per cent. Fe (31 mg. haemin of 8·41 per cent. Fe) in 1 litre of 1 per cent. sodium cyanide gives the same colour as 15·6 g. of haemoglobin treated first with hydrochloric acid and then with excess sodium cyanide, at a final dilution of 1 in 200 (King & Gilchrist, 1947).

PROCEDURES

Cyan Haematin. *Macro Method.* 0·5 ml. of blood are treated in a 100 ml. flask with 75 ml. N/10 HCl and left till the transformation to acid haematin appears to be complete, e.g. 5 to 10 minutes. Thereupon 20 ml. of 5 per cent. NaCN solution are added and water to the mark. The colour is read against the cyan-haematin standard with a green light filter.

Micro Method. 0·05 ml. blood are added to 4·95 ml. N/10 HCl, left for 5 to 10 minutes, and then 5 ml. of 2 per cent. NaCN added.

Alkaline Haematin. *Method 1.* 0·05 ml. of blood is diluted with 4·95 ml. of N/10 NaOH, heated in a boiling-water

bath for 4 to 5 minutes, cooled, and read against the haemin standard in a suitable colorimeter (Dubosecq, photoelectric, etc.), using a green light filter.

Method 2. (Method of Wu; cf., Peters and Van Slyke, 1932.) 0·05 ml. of blood is treated with 4 ml. of N/10 HCl, left at room temperature for 40 minutes, and then diluted to 5 ml. with N-NaOH.

Approximate Method with the Lovibond Comparator. Through the co-operation of Mr. G. S. Fawcett a Lovibond disc has been prepared to match the alkaline haematin colours. The values are spaced at 10 per cent. of the Haden normal—i.e., at differences of 1·56 g. haemoglobin per 100 ml. The blood is treated by either of the above procedures, the test-tube is placed in the Lovibond comparator, and the disc is rotated until a colour match is obtained. By careful comparison it is possible to gauge colours intermediate between those in the disc and hence to read the haemoglobin to within about 5 per cent.

Cyan-methaemoglobin Method. 0·05 ml. blood is collected in 9·85 ml. N/20 NH_3; 0·05 ml. of 4 per cent. $K_3Fe(CN)_6$ is added, and, after 15 minutes, 0·05 ml. of 8 per cent. NaCN solution. (The 0·05 ml. of $K_3Fe(CN)_6$ and NaCN are most conveniently and safely added from rubber-teated droppers, which are selected to give the correct quantity by counting the drops required to fill a 5 ml. cylinder.) The solution is mixed and the red colour measured in a visual or photoelectric colorimeter using a green light filter and a grey screen.

Carboxyhaemoglobin Method. 0·05 ml. blood is mixed with 9·95 ml. N/20 NH_3, well gassed with CO, and read against a grey screen with a green light filter.

The volumes 4·95, 9·85 and 9·95 ml. are best measured by means of specially calibrated pipettes. These are easily prepared by selecting good 5 and 10 ml. delivery pipettes and allowing 0·05 ml. or 0·15 ml. to run from the tip through a short, fine rubber connexion into a capillary pipette of appropriate size. The position of the meniscus is then noted, slightly below the original mark on the 5 or 10 ml. pipette, and a new mark is made with a file or diamond.

Ilford Neutral Grey Screen as Standard. The 0·5 density grey screens are of a suitable optical density for easy matching when used in a Dubosecq colorimeter with a spectrum green filter (both obtainable from Messrs. Ilford Ltd.). The

optical density of any screen supplied may not be quite that which was ordered, but the density is always stated exactly.

With Duboscq colorimeters the screen (1 in. diameter) is placed on the left-hand rack in place of the standard cup, and the rack adjusted so that the screen is against the bottom of the plunger. The green light filter ($\frac{3}{4}$ in. diameter) is placed over the eye-piece. The coloured solution is placed in the cup on the right-hand side, and its depth is adjusted until a match is secured. Several readings are made and the average depth in mm. recorded.

In Table 4 are given the readings for test solutions prepared from six normal bloods according to the three colorimetric methods, and read against the grey screen on three different colorimeters of the makes most commonly in use. The same grey screen was used, but different spectrum green light filters. The same readings were obtained with all three instruments for any blood treated by any one method. The readings have all been calculated to a common basis—i.e., the depth in mm. at which the bloods, whose O_2-capacities had been accurately determined by Van Slyke, would match the grey screen when diluted to a haemoglobin content of 15·6 g. corresponding to 20·9 ml. O_2.

TABLE 4.—*Ilford Neutral Grey Screen Equivalents of Colorimetric Haemoglobin Methods*

(Duboscq colorimeters, 0·57 density grey screen and Ilford spectrum green filter; average readings for 10 bloods.)

	Alk. haematin			Cyanmet-Hb			Carboxy-Hb		
	1	2	3	1	2	3	1	2	3
Av. for each colorimeter (mm).	10·05	10·00	9·88	14·57	14·53	14·70	14·73	14·77	14·78
Av. for all colorimeters (mm).		9·98			14·60			14·76	
Coefficient of variation (%)		3·05			1·95			2·48	
Equiv. of 0·50 density grey screen (mm).*	$9·98 \times \frac{0·50}{0·57}$			$14·60 \times \frac{0·50}{0·57}$			$14·76 \times \frac{0·50}{0·57}$		
		$= 8·75$			$= 12·8$			$= 12·95$	

1. Bausch and Lomb colorimeter, macro cups and plungers.
2. Bausch and Lomb biological type colorimeter, micro cups and plungers.
3. Klett colorimeter, macro cups and plungers.

* A 0·50 density screen would match a blood of 15.6 g. Hb/100 ml. when the blood is set at the calculated depth shown above, and may replace a standard blood of this strength when used in the colorimetric method with the spectrum green filter.

CALCULATION

To calculate the Hb content of any blood measured by one of the procedures described, the reading in mm. of the test solution against a 0·5 D screen is introduced in the equations as follows :

(1) **Alkaline Haematin**—

$$\frac{15{\cdot}6 \times 8{\cdot}75}{\text{reading of test}} = \text{g. Hb per 100 ml. blood}$$

or

$$\frac{100 \times 8{\cdot}75}{\text{reading of test}} = \text{Hb as percentage of Haden normal}$$

(2) **Cyan-methaemoglobin**—

$$\frac{15{\cdot}6 \times 12{\cdot}8}{\text{reading of test}} = \text{g. Hb per 100 ml. blood}$$

or

$$\frac{100 \times 12{\cdot}8}{\text{reading of test}} = \text{Hb as percentage of Haden normal}$$

(3) **Carboxyhaemoglobin,** similarly, using 12·95 mm. as standard reading.

With grey screens of other densities the standard equivalents in mm. are derived by simple calculation as illustrated in Table 4. For bloods of very low Hb content it is advisable to use a weaker grey screen—e.g., of 0·25 density, whose Hb equivalent would be half that of the 0·5 density screen. It is recommended that each worker should always calibrate his own grey screens against accurately analysed bloods.

With photoelectric colorimeters the neutral grey screen is also a satisfactory standard. It is necessary to calibrate a grey screen in a given instrument against several bloods whose Hb contents have been accurately determined. Once this calibration has been made the grey screen serves as a convenient and apparently permanent standard.

Chapter III

PROCEDURES FOR PLASMA

TAKING OF BLOOD FOR PLASMA

With a *dry* syringe blood is taken from an arm vein in the usual way. The arm should be warm ; if a tourniquet is applied to distend the vein, it should be used immediately before the puncture is made and removed as soon as blood begins to flow. Five to ten ml. of blood are taken and allowed to flow gently into a test-tube or small jar which has been dried in an oven after the addition of 1 drop of a 30 % solution of potassium oxalate. The blood is thoroughly mixed, by repeated inversion (not violent shaking), with the oxalate. If the plasma is needed for CO_2-combining power or chloride analysis, the blood must be centrifuged immediately, and in any case the plasma should be separated and pipetted off from the cells as soon as possible, without chilling of the blood.

PLASMA PROTEINS

The total quantity of protein in blood plasma varies in normal individuals from approximately 6 to 8 g. per 100 ml. Plasma protein is divided into two main fractions : globulin and albumin. Globulin includes fibrinogen. Normally, the approximate amounts of the proteins in plasma are albumin 3·4–6·0 g. per 100 ml. ; globulin (excluding fibrinogen) 1·5–3·0 g. per 100 ml. ; fibrinogen 0·2–0·4 g. per 100 ml. Where there is decrease of plasma protein—e.g., through proteinuria or malnutrition—the albumin is chiefly affected, and there is often a reduction of the albumin-globulin ratio (normally 1·3–4·0). A reduction of this kind is characteristic of nephrosis. An increase in the globulin, especially fibrinogen, may accompany inflammatory conditions.

Nesslerization Method

PRINCIPLE

Oxalated plasma diluted with isotonic sodium chloride is used for estimation of total protein. Another portion of the diluted plasma is treated with calcium chloride, and the fibrin clot removed. A further (fresh) sample of plasma is treated with sodium sulphite solution, which precipitates the "globulin," and the filtrate is used for estimation of "albumin." For total protein, and "albumin," the protein is precipitated with zinc sulphate and sodium hydroxide, the precipitates and the fibrin clot being then digested with sulphuric acid and selenium dioxide. The protein nitrogen is estimated colorimetrically, as ammonium sulphate, with Nessler's solution. The nitrogen figures multiplied by 6·25 give the approximate protein values, which are expressed as grams per 100 ml. of plasma.

METHOD

(A) Total Protein. 0·2 ml. of plasma (from oxalated blood) in a 20 ml. volumetric cylinder is diluted to 20 ml. with isotonic (0·9 g. per 100 ml.) sodium chloride. 0·5 ml. of this solution ($\equiv$ 0·005 ml. of plasma) is pipetted into 4 ml. of water in a Pyrex centrifuge tube. 0·1 ml. of zinc sulphate and 0·1 ml. of 0·5 N-sodium hydroxide are added with mixing, and the precipitate is centrifuged down.

When the supernatant liquid has been carefully decanted, the inverted tube is drained on a filter paper. 0·2 ml. of 50 per cent. sulphuric acid containing 1 per cent. of selenium dioxide is added, together with a small piece of porous pot. The mixture is gently boiled until blackening occurs and white acid fumes appear.* Heating is continued until the mixture is colourless, and for 3 or 4 minutes more. To the cooled colourless solution are added 5 ml. of water and the mixture is well shaken. Three ml. of Nessler's solution are added and the mixture again well shaken. The colour is compared with a standard prepared from 5 ml. of the standard

* An electric heater of the type supplied by Messrs. Gambrell Bros., Scientific Instruments, London, S.W. 18, and Messrs. Gallenkamps, Sun Street, London, E.C. 2, is useful for this purpose.

ammonium chloride solution (containing 0·01 mg. of nitrogen per ml.) and 3 ml. of Nessler's solution. A violet light filter may be used with advantage in this colorimetric comparison.

CALCULATION

$$\text{Total protein (A)*} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}05 \times \dfrac{100}{0{\cdot}005} \times \dfrac{6{\cdot}25}{1000} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 6{\cdot}25 \end{cases}$$

* g. per 100 ml. of plasma.

NOTE.—If the highest accuracy in the total protein estimation is not necessary, the 0·5 ml. of diluted plasma may be digested with sulphuric acid without the preliminary precipitation of the protein with zinc hydroxide. The results will be approximately 0·15 per cent. too high. 0·15 per cent. protein corresponds to the average non-protein nitrogen equivalent of 24 mg. per 100 ml. of normal plasma. This simplification should only be used in cases where the N.P.N. is known not to be elevated.

(B) Fibrin. To 10 ml. (= 0·1 ml. of plasma) of the solution of plasma in isotonic sodium chloride, placed in a narrow tube, is added 0·2 ml. of calcium chloride solution. The mixture is kept at 37° C. until clotting occurs. The fibrin is carefully collected on a thin glass rod, pressed to remove liquid, washed with water, and dropped into a test-tube for digestion. This, and also the colorimetric estimation, is carried out exactly as in the case of total protein.

CALCULATION

$$\text{Fibrin (B)} \dagger \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}05 \times \dfrac{100}{0{\cdot}1} \times \dfrac{6{\cdot}25}{1000} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}3125 \end{cases}$$

† g. per 100 ml. of plasma.

(C) **"Albumin."** 0·2 ml. of plasma is placed in a 10 ml. volumetric flask containing a micro drop of caprylic alcohol. The volume is made to 10 ml. with a solution of sodium sulphite. The mixture is kept at room temperature for 10 minutes and is then filtered through a fine filter paper. 0·5 ml. of the filtrate (≡ 0·01 ml. of plasma) is placed in 4 ml. of water in a Pyrex centrifuge tube and the protein precipitated with zinc sulphate and sodium hydroxide, spun, drained, digested, and estimated as ammonium sulphate exactly as in the case of total protein.

CALCULATION

$$\text{"Albumin" (C) †} \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}05 \times \dfrac{100}{0{\cdot}01} \times \dfrac{6{\cdot}25}{1000} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 3{\cdot}125 \end{cases}$$

$$\text{D † "globulin"} \begin{cases} = \text{Total protein} - \text{fibrin} + \text{"albumin"} \\ = \text{A} - (\text{B} + \text{C}) \end{cases}$$

† g. per 100 ml. of plasma.

SOLUTIONS

Fifty per cent. Sulphuric Acid, containing 1 per cent. of Selenium Dioxide (SeO_2), *Nessler's Reagent*, and *Standard Ammonium Chloride* as for urea and non-protein nitrogen.

Calcium Chloride.—2·5 g. per 100 ml. in water.

Sodium Sulphite.—42 g. of sodium sulphite ($Na_2SO_3.7H_2O$) dissolved in warm water and made to 100 ml.

It has been shown by Campbell and Hanna (1937) that treatment of plasma with sodium sulphite, instead of sodium sulphate (Howe, 1921) solution, enables the "globulin" fraction to be precipitated in 10 minutes (instead of 3 hours) and at room temperature (instead of at 37°C). This method, modified for use with 0·2 ml. of plasma, has been found, with bloods containing normal and abnormal ratios of albumin and globulin, to give results almost identical with those obtained by the sodium sulphate procedure previously described.

PLASMA PROTEINS

Kjeldahl Method

PRINCIPLE

The plasma proteins are precipitated by molybdic acid and the precipitate is digested with sulphuric acid. By this process the protein nitrogen is turned into ammonium sulphate. The digested mixture is transferred to a distillation apparatus and the ammonia is liberated by adding an excess of sodium hydroxide. The ammonia is distilled by steam and is carried over into an excess of standard sulphuric acid solution. By titration of the excess of standard acid, that amount of it which has been neutralized by the ammonia is determined. From this the percentage of proteins is calculated.

The albumin, and by difference the globulin, is determined in a similar manner in the filtrate from a sample of plasma which has been treated with sodium sulphite solution. Treatment of the solution with sodium sulphite brings about precipitation of the globulin fraction and leaves the albumin in solution.

METHOD

Total Protein. 0·2 ml. plasma (from oxalated blood) is mixed with 5 ml. of water in a round-bottom Pyrex centrifuge tube. To this mixture are added 0·2 ml. of 7·5 per cent. sodium molybdate and 0·2 ml. of $\frac{2}{3}$ N-sulphuric acid. The tube is shaken and centrifuged for 5 min. The supernatant fluid is completely decanted off and the tube inverted and allowed to drain on a filter paper. Two ml. of 50 per cent. sulphuric acid (containing 1 per cent. selenium dioxide) are added, together with a small piece of porous pot. The mixture is heated on an electric coil heater or with a very small gas flame and is gently boiled until blackening occurs ; thereafter the heating is continued for about 2 hours. If a condensate of selenium (reddish-brown deposit) forms at the side of the tube, it is returned to the body of the digestion mixture by gentle shaking.

The distillation of the ammonia is performed with the micro-Kjeldahl distillation apparatus as described under the method for total nitrogen in urine. The apparatus is cleaned by passing steam through it for about 20 minutes, and the cooled contents of the digestion tube are then transferred quantitatively into the apparatus with several washings of water.

Fifteen ml. of N/70 sulphuric acid containing the special indicator * are placed in a conical flask which is put in position with the end of the delivery tube under the surface of the acid. Five ml. of strong sodium hydroxide (40 per cent.) are now added to the distillation apparatus through a small funnel. The ammonia which is liberated is carried over into the standard acid by bubbling steam through the mixture. A burner is placed under the large round-bottom flask which is about half-full of water, and steam is passed through the mixture until the amount of liquid in the conical flask is about twice what it was at the beginning of the distillation (10 to 15 minutes). The conical flask is now lowered until the end of the condenser tube no longer dips into the liquid ; and the distillation is continued for a minute. The tube is washed down with water, to remove any adhering acid into the conical flask.

The titration of the excess acid in the conical flask is performed from a 10 ml. burette with N/70 sodium hydroxide. The titration figure so obtained is subtracted from the 15 ml. of N/70 sulphuric acid to give the ml. of standard acid which have been neutralized by the ammonia.

Cleaning of Distillation Apparatus. The Bunsen burner is removed from under the boiling flask and the partial vacuum thus created causes the liquid in the distillation apparatus to be sucked back into the waste chamber. When this has occurred the Bunsen burner is replaced and the steam allowed to pass until about 10 ml. of water have accumulated in the distillation chamber. The Bunsen burner is again removed and the distillate sucks back into the waste chamber. This

* N/70 sulphuric acid in 20 per cent. ethyl alcohol, containing 10 ml. per litre of Tashiro's indicator (0·08 per cent. methyl red and 0·02 per cent. methylene blue in alcohol).

process is repeated two or three times to ensure thorough cleansing of the distillation chamber. For the precautions which should be observed in performing the micro-Kjeldahl procedure, Pregl's " Quantitative Organic Microanalysis," 3rd ed., p. 88, should be consulted.

CALCULATION

1 ml. N/70 H_2SO_4 = 0·2 mg. of nitrogen

$$\text{Total Protein } * \begin{cases} = \text{ml. N/70 } H_2SO_4\dagger \times 0{\cdot}2 \times \dfrac{100}{0{\cdot}2} \times \dfrac{6{\cdot}25}{1000} \\ = \text{ml. N/70 } H_2SO_4 \times 0{\cdot}625 \end{cases}$$

* g. per 100 ml. of plasma.
† neutralized by the ammonia.

Albumin. 0·5 ml. of plasma and a small drop of caprylic alcohol are placed in a 10 ml. volumetric flask or cylinder and 42 per cent. sodium sulphite solution is added to the mark. The mixture is well mixed and allowed to stand for 15 minutes, when it is filtered through a Whatman No. 32 or 42 filter paper. Five ml. of filtrate (= 0·25 ml. of plasma) are transferred to a round bottom Pyrex centrifuge tube. A drop of caprylic alcohol and 1 ml. of 50 per cent. sulphuric acid are added. The tube is shaken to drive off the SO_2 liberated from the sodium sulphite by the sulphuric acid. 0·5 ml. of 7·5 per cent. sodium molybdate is added to precipitate the albumin and the tube is shaken and centrifuged. The supernatant fluid is carefully decanted off and the tube drained. The subsequent analysis is carried out exactly as described for total protein.

CALCULATION

$$\text{Albumin } \ddagger \begin{cases} = \text{ml. N/70 } H_2SO_4\S \times 0{\cdot}2 \times \dfrac{100}{0{\cdot}25} \times \dfrac{6{\cdot}25}{1000} \\ = \text{ml. N/70 } H_2SO_4 \times 0{\cdot}5. \end{cases}$$

‡ g. per 100 ml. of plasma.
§ neutralized by the ammonia.

Fibrin. One ml. plasma together with 1 ml. of 2·5 per cent. calcium chloride solution and 25 ml. of 0·9 per cent. NaCl solution are mixed and kept at 37° for 30 minutes or until clotting has occurred. The clot is collected on a thin glass rod, pressed to remove liquid, washed with a little 0·9 per cent. NaCl solution and placed in a round-bottom Pyrex centrifuge tube for digestion as in the case of total protein.

CALCULATION

$$\text{Fibrin } * \begin{cases} = \text{ml. N/70 } H_2SO_4\dagger \times 0{\cdot}2 \times \dfrac{100}{1} \times \dfrac{6{\cdot}25}{1000} \\ = \text{ml. N/70 } H_2SO_4 \times 0{\cdot}125. \end{cases}$$

* g. per 100 ml. of plasma.
† neutralized by the ammonia.

PLASMA SODIUM

The sodium of the blood is concentrated in the plasma, very little being found in the red cells. In normal persons plasma sodium values lie between 325 and 350 mg. per 100 ml. In acute cases of Addison's disease the amount is lowered, when values approaching 250 mg. may be encountered ; in chronic cases it may be only slightly depressed.

PRINCIPLE

After precipitation of the plasma proteins by trichloroacetic acid the sodium in the filtrate is precipitated as sodium zinc uranyl acetate. After washing, this precipitate is treated with potassium ferrocyanide and the resulting plum red colour (uranyl ferrocyanide) is compared with that produced by a standard sodium chloride solution which has been treated in a similar fashion.

METHOD

This method is adapted from the procedure of Noyons (1939). To 0·5 ml. of serum or plasma are added 1·5 ml. of 7 per cent. trichloroacetic acid. The mixture is shaken

well and filtered after 5 minutes; 0·2 ml. of the filtrate (≡ 0·05 ml. plasma) is transferred to a centrifuge tube containing 1 ml. of absolute alcohol and 0·4 ml. of zinc uranyl acetate reagent. The contents are mixed and kept in the ice-box overnight;* they are then centrifuged for 15 minutes. The supernatant solution is decanted, the tube allowed to drain on a filter paper for 10 minutes, and the lip dried; 5 ml. of absolute alcohol saturated with sodium zinc uranyl acetate are added; the contents are mixed, by rotating the tube, centrifuged for 15 minutes and drained as before. The precipitate is then dissolved in 10 ml. of dilute acetic acid; 0·25 ml. of potassium ferrocyanide solution is added and after mixing the tube is allowed to stand in the dark for 5 minutes. The coloured solution is compared with that produced from a standard sodium chloride solution, 0·2 ml. (≡ 0·15 mg. Na) of which has been treated simultaneously in the same way as the deproteinized plasma. (Colours must be read within 15 minutes or a clouding may occur. Green light filter.)

CALCULATION

$$\text{Plasma sodium}\dagger \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0{\cdot}15 \times \dfrac{100}{0{\cdot}05} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of unknown}} \times 300 \end{cases}$$

† mg. per 100 ml. of plasma.

SOLUTIONS

Standard NaCl (containing 0·75 mg. Na per ml.).—191 mg. of analytical dry sodium chloride dissolved in 100 ml. in water in a volumetric flask.

Trichloroacetic Acid.—Seven g. per 100 ml. in water.

Zinc Uranyl Acetate Reagent.—Twenty g. of uranyl acetate, $UO_2(CH_3COO)_2.2H_2O$, 60 g. of zinc acetate, $Zn(CH_3COO)_2$.

* It is not possible to obtain complete precipitation of the sodium zinc uranyl acetate in a short time. A 2 hr. precipitation may be used, however, although the results will be less accurate.

$2H_2O$, and 60 ml. of glacial acetic acid are added to 320 ml. of distilled water and warmed gently until dissolved. After standing 24 hours the solution is filtered into a dark bottle and stored in the ice-box. The solution must be filtered immediately before use.

Saturated Alcoholic Sodium Zinc Uranyl Acetate.—Forty ml. of zinc uranyl acetate reagent are mixed with 50 ml. of 50 per cent. alcohol saturated with sodium chloride ; 100 ml. of absolute alcohol are added, and, after standing in the ice-box overnight, the supernatant solution is decanted. The precipitate is washed several times with alcohol, drained, dried and then shaken with 500 ml. of absolute alcohol. This is stored in the ice-box and filtered immediately before use.

Potassium Ferrocyanide.—Twenty g. dissolved in water and made to 100 ml.

Dilute Acetic Acid.—0·5 ml. glacial acetic acid made to 100 ml. with water.

BILIRUBIN

Normal blood contains small amounts of the yellow pigment bilirubin.* These quantities may be greatly increased in various types of jaundice.

PRINCIPLE

The plasma is treated with diazotized sulphanilic acid, with the addition of ammonium sulphate and alcohol to precipitate the protein. The red colour produced was originally compared colorimetrically against a standard solution of bilirubin, treated with diazotized sulphanilic acid. This is, however, difficult to obtain pure, and various artificial " permanent standards " have been devised. The most satisfactory is that containing methyl-red (*o*-carboxybenzene-azo-dimethylaniline—2·9 mg. per litre at *p*H 4·63) in sodium acetate buffer. The colour of this solution accurately

* There is no generally accepted range of normal values for blood bilirubin. The normal values determined by this method fall within the range 0·1 to 1·3 mg. per 100 ml. of plasma, with the majority of the values within the limits 0·3 to 0·8 (Vaughan and Haslewood, 1938).

matches the colour obtained when 0·1 mg. of bilirubin is treated with the diazo reagent in a final volume of 25 ml.

METHOD

One ml. of plasma is treated in a centrifuge tube with 0·5 ml. of diazo reagent,* 0·5 ml. of saturated ammonium sulphate, and finally 3 ml. of absolute ethyl alcohol. The mixture is stoppered, thoroughly mixed, allowed to stand for a few minutes, and filtered. Under these conditions the dilution of the plasma closely approximates to 1 in 4, allowance being made for the volume of the precipitate and for the change in volume when alcohol is added to water. The colour of the clear filtrate is compared with the standard mentioned above (= 0·1 mg. of bilirubin in a volume of 25 ml.) (Haslewood and King, 1937).

CALCULATION

$$\text{Bilirubin } \dagger \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}1 \times \dfrac{4}{25} \times \dfrac{100}{1} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 1{\cdot}6 \end{cases}$$

† mg. per 100 ml. of plasma.

It is frequently found that brownish or purplish tints produced in the reaction make colorimetric comparison with the artificial standard difficult. These extraneous colours (probably due to traces of substances other than bilirubin which react with the diazo reagent) may be eliminated by the use of a green light filter (see section on photometric measurement, p. 142). The green filter (Ilford spectrum green is an appropriate type) is placed over the eye-piece of the colorimeter and the reading made against the artificial standard. The adjustment is then made in a green field whose two halves are of exactly the same quality of colour and differ only in intensity. The reading is taken in the usual way.

* If the diazo reagent is carefully "layered" above the plasma, and the tube allowed to stand for a few moments, a positive "direct" reaction (if present) may be seen at the liquid junction.

SOLUTIONS

Stock Standard Methyl-red Solution.—0·29 g. of pure methyl-red is dissolved in glacial acetic acid and the volume made to 100 ml.

Methyl-red Standard (2·9 mg. per litre at *p*H 4·63).—1 ml. of the above standard is placed in a litre flask, together with 5 ml. of glacial acetic acid. Water is added, and 14·4 g. of crystallized sodium acetate are washed into the flask. When dissolution is complete, the volume is made to 1 litre with water.

The Diazo Reagent.—This is made by mixing two solutions, A and B.

Solution A is made by dissolving 1 g. of sulphanilic acid in 250 ml. of N-hydrochloric acid, and making the volume to 1 litre with water.

Solution B contains 0·5 g. of sodium nitrite in 100 ml. of aqueous solution.

The diazo reagent mentioned above is made freshly before use by mixing 0·3 ml. of solution B with 10 ml. of solution A.

CHLORIDE

The blood plasma of normal persons contains from 560 to 620 mg. of chlorides per 100 ml. (expressed as NaCl *). A decreased plasma chloride may occur in febrile conditions, particularly pneumonia, Addison's disease, and in cases of gastro-intestinal disturbances associated with vomiting or with diarrhoea.

Iodimetric Method

PRINCIPLE

The method is based on the reaction :

$$NaCl + AgIO_3 \longrightarrow AgCl + NaIO_3$$

Silver iodate in ammoniacal solution is added to the deproteinized filtrate of blood or plasma. The excess of silver

* The results may often be advantageously expressed in terms of Cl instead of NaCl. To accomplish this the figure obtained for mg. NaCl should be multiplied by 0·607 (Cl/NaCl = 35·5/58·5).

iodate, together with the silver chloride formed, is precipitated by the addition of acid, leaving in solution an amount of soluble iodate equivalent to the amount of chloride originally present. After the addition of potassium iodide, the amount of iodine set free from the soluble iodate is determined by titration with thiosulphate.

METHOD

0·2 ml. of plasma is added to 1 ml. of water. 0·4 ml. of zinc sulphate solution and 0·4 ml. of 0·5 N-sodium hydroxide are added and thoroughly mixed. The mixture is then centrifuged. One ml. of the supernatant liquid ($\equiv$ 0·1 ml. of plasma) is treated with silver iodate reagent (0·5 ml.) and after mixing, with 2 N-sulphuric acid (0·5 ml.). The mixture is shaken and filtered through a small fine paper. One ml. of filtrate ($\equiv$ 0·05 ml. of plasma) with the addition of 1 ml. of 1 per cent. potassium iodide, is titrated with 0·005 N-sodium thiosulphate, with starch as indicator (Haslewood and King, 1936).

CALCULATION

Chloride (as mg. NaCl per 100 ml. of blood) = 97·5 $\times$ titre.

SOLUTIONS

Preparation of Silver Iodate Reagent.—Silver iodate is prepared by mixing equimolecular solutions of silver nitrate and potassium iodate. The precipitate is filtered, washed with distilled water, dried in vacuo, and preserved in the dark. Two g. of the dried solid are dissolved in 100 ml. N-ammonia. Both silver iodate and its ammoniacal solution appear to decompose slightly when kept, with liberation of soluble iodate. Immediately before a series of determinations, therefore, 5 ml. of the stock (2 per cent.) ammoniacal silver iodate are acidified with 2 N-sulphuric acid (5 ml.) and centrifuged. The supernatant fluid is discarded and the iodate redissolved in 5 ml. of fresh N-ammonia.

Adsorption Indicator Method

PRINCIPLE

Certain fluorescent indicators (e.g., eosin, fluorescein) are adsorbed strongly on precipitated silver chloride, but are released into the solution on addition of the first excess of silver nitrate.

PROCEDURE

0·2 ml. of plasma is treated in a conical centrifuge tube with 0·8 ml. of water and 6 ml. of acetone. The tube is closed with a rubber cap, and is inverted gently to mix the contents. It is then centrifuged, with the rubber cap in place to avoid evaporation. Five ml. of the clear supernatant are pipetted off from the precipitated proteins, and are transferred to a $\frac{3}{4}$ in. test-tube for titration.

At the same time 0·2 ml. of a standard NaCl solution (600 mg. per 100 ml.) is treated similarly with 0·8 ml. of water and 6 ml. of acetone, and 5 ml. of the mixture removed for titration.

To the 5 ml. of plasma supernatant and the 5 ml. of standard are added 0·3 ml. of 0·05 per cent. dichlorofluorescein in alcohol. Silver nitrate (N/58·5) is added from a 2 ml. burette, with shaking, until the sudden change from greenish white to a pinkish colouration is obtained.

CALCULATION

$$\text{Plasma chloride (mg. NaCl per 100 ml.)} = \frac{\text{ml. } AgNO_3 \text{ for test}}{\text{ml. } AgNO_3 \text{ for standard}} \times 600$$

CARBON DIOXIDE COMBINING POWER

Between 55 and 75 ml. of carbon dioxide are normally held (partly in solution and partly in chemical combination) by 100 ml. of blood plasma. About 90 per cent. is present

in the combined form as sodium bicarbonate, and a determination of the amount of carbon dioxide which can be held by a sample of blood plasma gives a measure of the alkali present. The alkali of plasma is thus usually referred to in terms of its "CO_2-combining power." In clinical conditions in which an "acidosis" is present (e.g., diabetes, starvation, and severe nephritis) the production in the body of various acid substances results in part of the alkali of the plasma being combined with these other acid bodies. Less carbon dioxide is consequently held in combination by the plasma, and the "CO_2-combining power" is therefore reduced. In other clinical conditions involving the accumulation of excess alkali in the blood (e.g., alkali administration, intestinal obstruction, over-breathing) more carbon dioxide is held in combination in the plasma than is normally the case. This condition is usually known as one of "alkalosis."

PRINCIPLE

The carbon dioxide is liberated from its combination with alkali in the plasma by the addition of acid and the volume of gas evolved is measured in a special apparatus designed by Van Slyke. The volumetric method and apparatus introduced by Van Slyke and Cullen (1917) is described. This has been found more suitable for student use than the manometric apparatus of Van Slyke and Neill (1924). The latter should be used for work requiring the highest accuracy (cf., Peters and Van Slyke, 1932), but the simpler volumetric apparatus is adequate for all routine purposes.

METHOD

Five ml. of freshly drawn oxalated blood are centrifuged until the supernatant plasma is free of cells. About 2 ml. are transferred to a separatory funnel (about 250 ml.). By means of a short rubber tube, the stem of the funnel is connected to a bottle containing glass beads and a little water (see Fig. 1).

The stop-cock of the separatory funnel is opened, the stopper removed and a long breath slowly blown into the mouthpiece of the bottle and through the separatory funnel. Immediately the expiration is complete the stop-cock is closed and the stopper replaced in the separatory funnel. The plasma is now in contact with a carbon dioxide-containing atmosphere which is very close in composition to alveolar

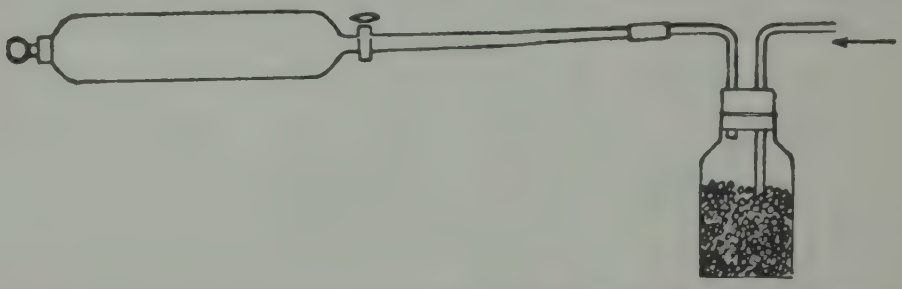


FIG. 1.—Apparatus for saturating plasma with CO_2 (Van Slyke). The bottle contains glass beads. (From Beaumont & Dodds: *Recent Advances in Medicine.*)

air. After disconnecting the funnel at the rubber tube, the plasma is spread over the walls of the funnel by gentle rotation for 1 min. in order that it may become saturated with the carbon dioxide of the alveolar air. The plasma is now allowed to drain into the narrow end of the funnel.

The Van Slyke apparatus (see Fig. 2) is prepared for use in the following manner.

Stop-cock (F) is opened and the mercury reservoir is raised. The mercury rises in the chamber (A) of the apparatus, and is allowed to flow into the side arm from the stop-cock (E). Stop-cock (E) is now turned to connect with the inlet chamber (B) and the level of mercury raised in order to fill the capillary opening at the bottom of (B). (E) is now turned off and the apparatus should be air free (e.g., with no bubbles at the top of the tubes (C) and (D) immediately below the stop-cock (F)). This can be tested for, with the stop-cock (F) open first to connect the chamber with (D) and then with (C), by lowering the mercury reservoir until the mercury falls to the 50 ml. mark and then gently raising till the mercury comes with a clicking sound into the top of the burette below (E). If the mercury does not click soundly

into the top of the burette, air has leaked into the apparatus and the stop-cocks should be re-greased in order that they shall be airtight.

Distilled water (1 ml.) is added to the inlet chamber (B). One ml. of plasma is removed from the separatory funnel and is run into the chamber (B) below the surface of the distilled water. A drop of caprylic alcohol is added as an anti-frothing agent and the contents of (B) are allowed to run into the burette by carefully opening the stop-cock (E) and very gradually lowering the mercury reservoir from its previous position at the height of (E). A small amount of water should be left filling the capillary at the bottom of (B). Stopcock (E) is now turned off, great care being taken that no air is allowed to pass through it into the burette. 0·5 ml. of lactic acid solution (1 vol. of conc. acid diluted to 10 vols. with water) is now placed in (B) and is carefully drawn into the burette in the manner already described, care being again taken to leave a small amount of liquid in the capillary at the bottom of (B) and admitting no air into the burette. The mercury reservoir is lowered until the mercury is at the 50 ml. mark and the stop-cock (F) is turned off. The contents of the burette and 50 ml. chamber are now under a partial vacuum and the carbon dioxide which has been liberated from its combination as sodium bicarbonate by the lactic acid is seen to boil off from the liquid. In order to bring all the carbon dioxide out of the acidified plasma the apparatus is gently shaken by inversion about twenty times, after carefully removing it from the clamp. After replacing the apparatus, the stop-cock (F) is opened and the plasma mixture very carefully sucked into the chamber (D), leaving

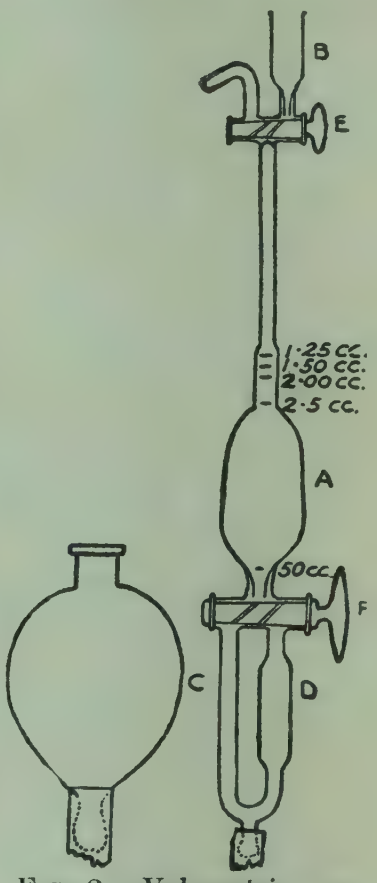


FIG. 2.—Volumetric van Slyke apparatus.
(After Beaumont and Dodds)

TABLE 5.—*For Calculation of the CO_2-combining Power of Plasma*

(v = measured ml. of CO_2) (p = barometic pressure)

$v \frac{p}{760}$	ml. CO_2 (reduced to 0° and 760 mm.) bound bicarbonate in 100 ml. of plasma.				$v \frac{p}{760}$	ml. CO_2 (reduced to 0° and 760 mm.) bound bicarbonate in 100 ml. of plasma.			
	15°	20°	25°	30°		15°	20°	25°	30°
0·20	9·1	9·9	10·7	11·8	0·60	47·7	48·1	48·5	48·6
1	10·1	10·9	11·7	12·6	1	48·7	49·0	49·4	49·5
2	11·0	11·8	12·6	13·5	2	49·7	50·0	50·4	50·4
3	12·0	12·8	13·6	14·3	3	50·7	51·0	51·3	51·4
4	13·0	13·7	14·5	15·2	4	51·6	51·9	52·2	52·3
5	13·9	14·7	15·5	16·1	5	52·6	52·8	53·2	53·2
6	14·9	15·7	16·4	17·0	6	53·6	53·8	54·1	54·1
7	15·9	16·6	17·4	18·0	7	54·5	54·8	55·1	55·1
8	16·8	17·6	18·3	18·9	8	55·5	55·7	56·0	56·0
9	17·8	18·5	19·2	19·8	9	56·5	56·7	57·0	56·9
0·30	18·8	19·5	20·2	20·8	0·70	57·4	57·6	57·9	57·9
1	19·7	20·4	21·1	21·7	1	58·4	58·6	58·9	58·8
2	20·7	21·4	22·1	22·6	2	59·4	59·5	59·8	59·7
3	21·7	22·3	23·0	23·5	3	60·3	60·5	60·7	60·6
4	22·6	23·3	24·0	24·5	4	61·3	61·4	61·7	61·6
5	23·6	24·2	24·9	25·4	5	62·3	62·4	62·6	62·5
6	24·6	25·2	25·8	26·3	6	63·2	63·3	63·6	63·4
7	25·5	26·2	26·8	27·3	7	64·2	64·3	64·5	64·3
8	26·5	27·1	27·7	28·2	8	65·2	65·3	65·5	65·3
9	27·5	28·1	28·7	29·1	9	66·1	66·2	66·4	66·2
0·40	28·4	29·0	29·6	30·0	0·80	67·1	67·2	67·3	67·1
1	29·4	30·0	30·5	31·0	1	68·1	68·1	68·3	68·0
2	30·3	30·9	31·5	31·9	2	69·0	69·1	69·2	69·0
3	31·3	31·9	32·4	32·8	3	70·0	70·0	70·2	69·9
4	32·3	32·8	33·4	33·8	4	71·0	71·0	71·1	70·8
5	33·2	33·8	34·3	34·7	5	71·9	72·0	72·1	71·8
6	34·2	34·7	35·3	35·6	6	72·9	72·9	73·0	72·7
7	35·2	35·7	36·2	36·5	7	73·9	73·9	74·0	73·6
8	36·1	36·6	37·2	37·4	8	74·8	74·8	74·9	74·5
9	37·1	37·6	38·1	38·4	9	75·8	75·8	75·8	75·4
0·50	38·1	38·5	39·0	39·3	0·90	76·8	76·7	76·8	76·4
1	39·1	39·5	40·0	40·3	1	77·8	77·7	77·7	77·3
2	40·0	40·4	40·9	41·2	2	78·7	78·6	78·7	78·2
3	41·0	41·4	41·9	42·1	3	79·7	79·6	79·6	79·2
4	42·0	42·4	42·8	43·0	4	80·7	80·5	80·6	80·1
5	42·9	43·3	43·8	43·9	5	81·6	81·5	81·5	81·0
6	43·9	44·3	44·7	44·9	6	82·6	82·5	82·4	82·0
7	44·9	45·3	45·7	45·8	7	83·6	83·4	83·4	82·9
8	45·8	46·2	46·6	46·7	8	84·5	84·4	84·3	83·8
9	46·8	47·1	47·5	47·6	9	85·5	85·3	85·2	84·8
0·60	47·7	48·1	48·5	48·6	1·00	86·5	86·2	86·2	85·7

a very small amount of fluid at the bottom of the 50 ml. chamber. (F) is now turned round so as to be open to (C) and the mercury is allowed to run into the 50 ml. chamber and up into the graduated burette tube. The surface of the mercury in the reservoir is held level with the surface of the mercury in the burette tube, so that the carbon dioxide in the burette is now at a pressure equal to that of the atmosphere. The volume of the gas is read from the burette and is corrected to standard pressure and temperature. For this calculation the temperature and barometric pressure at the time of the experiment are, of course, necessary.

CALCULATION

The figure obtained, the ml. of carbon dioxide released from 1 ml. of plasma, is reduced by reference to the table to the number of ml. of carbon dioxide which would be liberated from 100 ml. of plasma at standard temperature and pressure. This is the " CO_2-combining power " of the plasma.

PHOSPHORUS : DISTRIBUTION IN BLOOD

The phosphorus of the blood is present partly as inorganic phosphate and partly in combination with several organic substances. It is distributed as follows :

	Whole blood	Plasma
	mg. P per 100 ml.	
Inorganic phosphate . . .	2 to 3	2 to 3
Ester ,, . . .	20 to 30	1 to 2
Lipide ,, . . .	11 to 14	7 to 10
Nucleotide ,, . . .	2 to 3	—
Total ,, . . .	35 to 50	10 to 15

The inorganic phosphate is about equally distributed in cells and plasma. The ester phosphate consists of " organic salts " of phosphoric acid, which are present, for the most part, in the cells. Lipide phosphate is that contained in the phosphatides—lecithin, kephalin and sphingomyelin. There is about twice as much lipide phosphate in the cells as in the plasma.

Ester Phosphate. The phosphoric esters, like inorganic phosphate, are soluble in acid and are present in a filtrate of blood which has been deproteinized with trichloroacetic acid. The inorganic and ester phosphates are known as "the acid soluble phosphate." Only a small amount of ester phosphate is present in the plasma (about 1 mg. P per 100 ml.); it is thought to be a phosphoric ester of a sugar or related substance. The cells contain 40–60 mg. ester P per 100 ml. Much of it is present as phospho-glyceric acid; the composition of the remainder is not fully known. Some of these esters are readily hydrolyzed with liberation of free phosphate by the action of the enzymes present in the blood. Consequently, an increase in the inorganic phosphate occurs when blood is allowed to stand; this change is accelerated if the cells be haemolyzed. It is, therefore, essential to conduct the analysis for free and ester phosphate on freshly drawn blood.

The ester phosphate is definitely low in rickets. It returns to normal on the addition of Vitamin D to the diet. Children suffering from osteomyelitis may have a high ester phosphate of the plasma, with that of the cells unaffected or even decreased. An increase of ester phosphate has been observed in conditions of trauma due to resorption from muscle.

PRINCIPLE

Inorganic phosphate couples with molybdic acid to form a yellow phospho-molybdate; this can be reduced to give a blue colour which is directly proportional to the amount of inorganic phosphate present. The ester phosphate is not capable of reacting with molybdic acid until after destruction of the organic matter by digestion with hot concentrated perchloric acid, when all the phosphate is converted to the inorganic, reactable form. Its measurement gives the total phosphate, inorganic plus ester, which was originally present in the trichloroacetic acid extract of the blood.

The ester phosphate is obtained from the difference between the inorganic phosphate and the total "acid-soluble" phosphate.

METHOD

Two ml. of freshly drawn oxalated plasma or whole blood are diluted with 5 ml. of water and treated with 3 ml. of 25 per cent. trichloroacetic acid. The mixture is well shaken and is filtered after 5 minutes. Analyses for total "acid-soluble" and inorganic phosphate are carried out on the filtrate as below.

Total "Acid-soluble" Phosphate. Five ml. of the trichloroacetic acid filtrate from plasma (≡ 1 ml. plasma) or 0·5 ml. of filtrate from whole blood or cells (≡ 0·1 ml. blood) are measured into a 15 ml. volumetric flask of good acid-resistant glass, 1·2 ml. of 60 per cent. perchloric acid are added and a small piece of porous pot to prevent bumping. The contents of the flask are heated carefully with a micro-burner (using a very small flame) or on an electric heater. (Approximately 0·2 ml. of perchloric acid is lost in the heating.) As the contents of the tube become concentrated they turn brown and then, as the temperature rises and the acid begins to fume, they become colourless, the organic matter being completely oxidized in a few minutes. In some cases, where the amount of organic material is large and the oxidation slow, it may be necessary to add a drop of nitric acid or of 30 per cent. hydrogen peroxide; in this case it will be necessary to continue the heating for 3 or 4 minutes after the mixture has become colourless, in order to drive off the excess of these reagents. The cooled contents are diluted with about 10 ml. of water. One ml. of 5 per cent. molybdate and 0·5 ml. of reducing agent are added to the test. At the same time two standards are prepared from 5 ml. and 10 ml. of the standard solution (0·01 mg. P per ml.), 1 ml. of perchloric acid, 1 ml. of molybdate and 0·5 ml. of reducing agent. Test and standards are diluted to the mark, mixed, and the test read after 10 minutes against the appropriate standard, using a red or orange light filter (e.g., Ilford spectrum red or orange).

CALCULATION

$$\text{Total "acid-soluble" phosphate}^{*} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times C \times 0{\cdot}01 \times \frac{100}{x}$$

* mg. P per 100 ml.

Where C is the number of ml. of the *standard solution* and x is the number of ml. of plasma, whole blood or cells represented in the trichloroacetic acid filtrate used in the test.

Inorganic Phosphate. Five ml. of the clear filtrate (≡ 1 ml. of plasma or blood) are transferred to a 15 ml. volumetric flask. In another 15 ml. flask are placed 5 ml. of the standard phosphate solution (≡ 0·05 mg. P). Water is added to each flask, followed by 1 ml. of perchloric acid, 1 ml. of 5 per cent. molybdate, 0·5 ml. of the reducing agent and water to the 15 ml. mark. The contents of the flasks are gently shaken between each addition, and finally mixed by inverting and shaking. The colours are read after 10 minutes.

CALCULATION

$$\text{Inorganic phosphate}^{\dagger} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}05 \times \frac{100}{1}$$

† mg. P per 100 ml.

Ester phosphate = total "acid-soluble" phosphate *minus* inorganic phosphate.

Lipide Phosphate. Lipide phosphate is often determined in connection with investigations of fat metabolism. Increases have been noted in diabetes and nephritis, where the lipide phosphate is related to the degree of lipemia, and in pregnancy and certain hepatic conditions.

METHOD

One ml. of plasma or whole blood is added drop-wise, with shaking, to 15 ml. of a mixture of 90 parts absolute alcohol and 10 parts ether in a 25 ml. volumetric flask. The mixture

is heated in a hot water bath until it is boiling. It is then cooled, made to volume with alcohol-ether and is thoroughly shaken. The mixture is filtered, and 10 ml. of filtrate are carefully evaporated to dryness (in two 5 ml. portions to minimize the chance of loss through frothing) in a 15 ml. flask. The phosphate is estimated by digestion with perchloric acid, as in the method for total acid-soluble phosphate.

Total Phosphate in Blood. The whole of the phosphate of the blood can be determined by the colorimetric method after destruction of the organic matter (proteins, fats, etc.) by boiling perchloric acid. For this purpose 0·1 ml. of blood, accurately measured, is digested and the determination carried out as in the total acid-soluble phosphate method.

SOLUTIONS

Trichloroacetic Acid Solution.—Twenty-five g. of best grade trichloroacetic acid are dissolved in water and made to 100 ml.

Ammonium Molybdate Solution.—Five g. ammonium molybdate dissolved in water and made to 100 ml.

Reducing Agent. 0·2 per cent. 1 : 2 : 4-aminonaphtholsulphonic acid in 12 per cent. sodium meta-bisulphite and 2·4 per cent. sodium sulphite (w/v).

The reducing agent may be prepared fresh for use from a tablet containing the correct amounts of 1 : 2 : 4-aminonaphtholsulphonic acid, sodium sulphite and sodium meta-bisulphite. A tablet is ground with 10 ml. of water, and the filtered solution is ready for use. (Obtainable from Messrs. Gallenkamp.)

Stock Standard Phosphate.—A stock solution is made by dissolving 2·194 g. of pure potassium dihydrogen phosphate (KH_2PO_4) in 500 ml. in water. This solution contains 1·0 mg. P per ml.

The Standard Solution is made by diluting 5 ml. of the stock solution to 500 ml. with water. This solution contains 0·01 mg. P per ml. Both solutions should be kept saturated with chloroform to prevent any bacterial growth, which might otherwise cause a loss of inorganic phosphate.

PHOSPHATASE IN SERUM OR PLASMA

Phosphatase is the name given to an enzyme shown to be present in bone and ossifying cartilage by Robison (1923). He has demonstrated that this enzyme is intimately related with the process of bone formation. The blood plasma of normal individuals contains small amounts of phosphatase corresponding to about 5 to 10 " units "—the arbitrary term in which the amount of the enzyme is expressed. In conditions of generalized bone disease the phosphatase appears to leak out of the bone into the blood, and appears there in large amounts. Its quantitative determination in the serum or plasma is of diagnostic value in cases of rickets, Paget's disease, osteitis fibrosa cystica (Kay, 1931) and in many cases of malignant bone disease (Franseen, Simmons and McLean, 1939). A marked accumulation of the enzyme in the serum has also been shown to take place in obstructive jaundice (Roberts, 1933, Armstrong, King and Harris, 1934). In conditions of toxic and infective jaundice the rise is not so marked, and in haemolytic jaundice the enzyme is present in normal amount. The range of values encountered clinically in the different types of jaundice is fully discussed by Herbert (1935). Normal persons have 10 or less units per 100 ml. Cases of haemolytic jaundice show values which are not above the normal. In infective and toxic jaundice, and in obstructive jaundice where the obstruction is only partial or intermittent, the values are raised above normal and are usually between 10 and 30 units. In obstructive jaundice where the obstruction is complete or of long standing, the phosphatase is greatly increased, values above 30 and sometimes as high as 200 being encountered. In generalized bone disease the values are very similar to those found in obstructive jaundice.

PRINCIPLE

The estimation of phosphatase depends upon measuring the amount of hydrolysis which takes place when the enzyme is allowed to act on a suitable substrate—an ester of phos-

phoric acid (such as phenyl phosphate)—under standard conditions. The amount of phosphate or phenol so liberated may be taken as the measure of the amount of enzyme present. The phenol is more easily determined than the phosphate, and three times as much phenol (by wt.) as phosphorus is set free. The hydrolysis is carried out at the optimum *p*H of 10 for 15 minutes. The results thus obtained agree very closely with those of the method of King and Armstrong (1934) of which this is a modification,* and with the method of Jenner and Kay (1932). The results are expressed in arbitrary " units " of phosphatase activity.

The " unit " of phosphatase is defined as the amount of the enzyme which will set free 1 mg. of phenol in the given time under the conditions of the test; and hence " units " per 100 ml. = mg. of phenol set free from the phenyl phosphate under the standard conditions.

METHOD

Test. In a conical centrifuge tube are placed 2 ml. of buffer and 2 ml. of substrate. The tube is allowed to remain in a water-bath at 37° C. for 3 minutes. Without removal of the tube from the bath, exactly 0·2 ml. of plasma (which must be cell-free) is added and mixed. The stoppered tube is allowed to remain in the bath exactly 15 minutes. At the end of this time 1·8 ml. of dilute Folin-Ciocalteau phenol reagent are added and the mixture centrifuged or filtered.

Control. In another tube are placed 2 ml. of buffer and 2 ml. of substrate. 1·8 ml. of dilute phenol reagent are added, followed by 0·2 ml. of plasma and the mixture centrifuged or filtered.

Four ml. of filtrate from the test and control solutions are pipetted into test-tubes. One ml. of 25 per cent. sodium carbonate is added and the tubes replaced in the water-bath for 5 minutes to bring up the colour.

Comparison. The solutions are compared in the colorimeter with a standard made up at the same time by taking 4 ml. of standard-phenol-solution-and-reagent and 1 ml. of 25 per cent. sodium carbonate. The test solution is placed

* The Bodansky unit is about one-third the King-Armstrong unit.

on the left-hand side of the colorimeter and set at 30 mm. The standard is placed on the right-hand side and the colours matched. The use of an orange or red filter will be found to increase the ease of colorimetric comparison, particularly with weak solutions.

CALCULATION

The phosphatase activity of a plasma is expressed as units per 100 ml. and is numerically equal to the mg. of phenol which would be set free from the phenyl phosphate under the standard conditions by 100 ml. of plasma. Thus :

Units of phosphatase per 100 ml. =

mg. phenol per 100 ml. plasma in test *minus* mg. phenol per 100 ml. plasma in control.

The number of mg. phenol in 100 ml. of plasma in the test and in the control is found by the equation :

$$\frac{\text{Reading of standard}}{\text{Reading of test}} \times \text{strength of standard} \times \frac{6}{4} \times \frac{100}{0{\cdot}2}$$

With the unknown solution set at 30 mm. and the strength of standard = 0·04 mg. this equation can be written :

$$\frac{\text{Reading of standard}}{30} \times 0{\cdot}04 \times \frac{6}{4} \times \frac{100}{0{\cdot}2}$$

All figures in the above equation cancel out, making it equal to " Reading of standard."

More simply then :

Units of phosphatase per 100 ml. =

Reading of standard against the test *minus* Reading of standard against the control.

SOLUTIONS

Buffer.—M/10 sodium carbonate-bicarbonate ($6Na_2CO_3 : 4NaHCO_3$). 6·36 g. anhydrous sodium carbonate and 3·36 g. sodium bicarbonate are dissolved in distilled water and made to 1 litre.

Substrate.—M/100 disodium phenyl phosphate. 2·18 g. dissolved in 1 litre in water. The solution should be brought quickly to the boil to destroy any organisms, cooled immediately and preserved with a little chloroform.

Phenol Reagent of Folin and Ciocalteau.—See p. 64; also Peters and Van Slyke's "Quantitative Clinical Chemistry," vol. ii, p. 655, and Beaumont and Dodd's "Recent Advances in Medicine," 8th ed., p. 403. This reagent is diluted 1 in 3.

Twenty-five per cent. Sodium Carbonate (w/v).—Twenty-five g. of anhydrous sodium carbonate are dissolved in warm water and made to 100 ml. This solution is preserved in a warm place, otherwise the sodium carbonate tends to crystallize out.

Stock Standard Phenol (100 mg. per 100 ml.).—One g. pure crystalline phenol is dissolved in, and made to 1 litre with 0·1 N-HCl.

Standard-Phenol-and-Reagent (1 mg. phenol per 100 ml.).—Five ml. of the stock standard phenol (100 mg. per 100 ml.) are accurately measured into a 500 ml. volumetric flask, 100 ml. of dilute (1 : 3) Folin-Ciocalteau reagent are added and water to the mark. This solution will keep at least six months, if preserved in the ice-chest.

ACID PHOSPHATASE

The prostate contains a very active phosphatase which, unlike the phosphatase of bone, intestine, kidney, etc., has its *p*H optimum at an acid reaction. To distinguish it from the better known "alkaline phosphatase," this enzyme is called "acid phosphatase." There is very little present normally in the blood, but in prostatic conditions, particularly in carcinoma of the prostate with secondaries in the bone, very large amounts may appear in the blood. These are derived in part from the prostate and in part from the secondary growths in the bone. There are normally 1–3 arbitrary acid phosphatase units in 100 ml. of serum or plasma. In cases of carcinoma of the prostate with secondaries, values much greater than this and up to 30 units and more have been observed (cf., Gutman and Gutman, 1938).

PRINCIPLE

The *p*H optimum of acid phosphatase is between 4·5 and 5 ; whereas that of alkaline phosphatase is between *p*H 8·4 and 10, depending on the substrate used. Because of this great difference in *p*H optimum, it is possible to estimate one phosphatase in the presence of the other merely by allowing it to act on the substrate at the characteristic optimum *p*H. Thus the alkaline phosphatase in blood plasma is inactive at the reaction where acid phosphatase works best ; and vice versa.

The same substrate, i.e., phenyl phosphate, is used for the acid phosphatase as for the alkaline, but citric acid-sodium citrate buffer of *p*H 4·9 is used instead of the alkaline sodium carbonate buffer.

METHOD

Two ml. of M/100 disodium phenyl phosphate are pipetted into each of two test-tubes together with 2 ml. of the buffer solution of *p*H 4·9. The tubes are allowed to remain in a 37° water-bath for 3 minutes to allow the contents to attain the temperature of the bath. After this time, 0·2 ml. of the plasma is added to one of the tubes and the enzymic hydrolysis allowed to proceed for exactly 1 hour. 1·8 ml. of Folin and Ciocalteau's phenol reagent are then added to each tube and 0·2 ml. of plasma to the control tube. The two tubes are shaken and centrifuged, and 4 ml. of the supernatant pipetted into two test tubes. After the addition of 1 ml. of 25 per cent. sodium carbonate, the tubes are replaced in the water-bath for 15 minutes to allow the colours to develop. The solutions are read in a Duboscq or photoelectric colorimeter against a standard solution of phenol (i.e., 4 ml. of standard-phenol-and-reagent and 1 ml. of sodium carbonate) which has been similarly treated. The results are expressed in terms of units which are equal to mg. of phenol liberated in 1 hour. The calculation is the same as for alkaline phosphatase.

The colours are rather pale for normal bloods, and for prostate cases where the acid phosphatase is not much

elevated. It may be preferable in these instances to use a somewhat longer incubation period, e.g., 3 hours. If this is done the mg. phenol liberated are of course divided by 3 to reduce the figure to 1 hour.

SOLUTIONS

See alkaline phosphatase for all solutions except the buffer. *Citric acid-sodium citrate buffer* *p*H 4·95 is prepared as follows :

Dissolve 21·0 g. of crystalline citric acid in water, add 188 ml. of N-NaOH, and make to 500 ml. The *p*H should be checked and adjusted to *p*H 4·9 if necessary, by dropwise addition of N-NaOH or N-HCl. This solution should be preserved with a few drops of chloroform and kept in the ice-chest.

N.B.—Acetate buffer may be used, but citrate is here adopted to make the method conform to Gutmans' 1940 procedure.

PLASMA AMYLASE

The determination of plasma amylase is of importance in the diagnosis of acute pancreatitis in which high values may be obtained. It has the advantage over the estimation of urinary diastase in that it is unnecessary to wait for the collection of a 24-hour specimen of urine. Normal values are given by Somogyi (1941) as 70–150 units per 100 ml.

PRINCIPLE

0·5 ml. of plasma is incubated at 37° with 1·5 mg. of starch and the time noted when the mixture no longer gives a blue colour with iodine solution.

The amylase activity is expressed in terms of " units " of amylase per 100 ml. The " unit " is defined as the amount of amylase which will destroy 1·5 mg. of starch in 8 minutes.*

* The unit is defined in this way so that the results become almost identical with those of Somogyi (1941) whose unit is defined in terms of the amount of reducing sugar liberated from starch by plasma under precisely stated conditions.

METHOD

Two ml. of starch solution are introduced into a test-tube which is placed in a water-bath at 37° for 2 minutes to allow the contents to attain the temperature of the bath. 0·5 ml. of plasma is added to the tube and a stop-watch started. At intervals of 2–5 minutes 0·2 ml. portions of the reaction mixture are withdrawn and added to previously prepared tubes containing 0·2 ml. samples of iodine solution and the colour observed. The time at which a blue colour is no longer obtained is noted and from this time the amylase activity is calculated. The exact time intervals must depend on experience, since by examination of the colour at each addition it is possible to judge when the next sample should be tested.

CALCULATION

$$\text{Amylase Activity} * = \frac{8}{t} \times \frac{100}{0{\cdot}5} = \frac{1600}{t}$$

* Units of amylase per 100 ml. of plasma where t is the time taken for the destruction of the starch.

SOLUTIONS

Starch.—Seventy-five mg. of starch are weighed out and made into a paste with a few ml. of cold water. This is poured into a solution of 250 mg. NaCl in 80 ml. of boiling water. The mixture is then cooled and made to 100 ml. Two ml. of this solution contain 1·5 mg. of starch.

Iodine.—Approximately N/20, made by fresh dilution of N/10.

PLASMA ASCORBIC ACID

The amount of ascorbic acid present in the blood plasma of apparently healthy individuals ranges from about 0·4 mg. to 2 mg. per 100 ml. The amount varies greatly with the nutritional habits of the individual. People consuming large quantities of citrus fruits, e.g., in California, have more ascorbic acid in their blood than those on a more usual type of diet. A large group of people in England, who appeared

healthy and showed no clinical signs of scurvy, had values ranging from 0·3 to 1·3 mg. with an average of 0·65. Only 2 per cent. of the cases were below 0·4 mg. (Young, Wood and King, 1943). Prunty and Vass (1943) considered the plasma ascorbic acid a reliable and satisfactory index of the nutritional state with respect to Vitamin C. The determination is more easily carried out than a saturation test (cf., urine ascorbic acid). A " state of saturation " is usually attained when the plasma ascorbic acid is 0·8 mg. per 100 ml. or greater.

PRINCIPLE

The oxidation-reduction dye dichlorophenol-indophenol is used to titrate a standard solution of pure ascorbic acid, which is prepared so as to be of about the same concentration as that of a deproteinized filtrate of blood plasma. The volumes of standard solution and of plasma filtrate used to decolorize a standard amount of the dye are then used to calculate the ascorbic acid concentration of the plasma.

METHOD

At least 5 ml. of oxalated blood are required. The plasma should not be separated until just before the preparation of the protein-free filtrate. Two ml. of plasma are diluted with 4 ml. of water and 4 ml. of 5 per cent. metaphosphoric acid, mixed and centrifuged. The clear supernatant fluid is then run (from a 5 ml. burette) into 0·05 ml. of dye until all trace of pink colour disappears. The titration should be made quickly so as to minimize the small amount of reduction of the dichlorophenol-indophenol which may take place due to traces of non-ascorbic acid reducing substances in the plasma filtrate. Any froth which might make the titration difficult can be cleared by touching with fine wire which has been dipped in caprylic alcohol.

To 2 ml. of the dilute standard solution are added 4 ml. of water and 4 ml. of 5 per cent. metaphosphoric acid. This is titrated against 0·05 ml. of the dye solution, conveniently contained in a conical centrifuge tube. The ascorbic acid solution is run into the dye from a 5 ml. burette until all trace

of pink colour has disappeared. The titration gives the ascorbic acid equivalent of 0·05 ml. of dye.

CALCULATION

$$\text{Ascorbic acid } * = \frac{\text{Titration of standard}}{\text{Titration of plasma filtrate}} \times 1{\cdot}2$$

* mg. per 100 ml. plasma.

SOLUTIONS

Preparation of Dye.—One hundred mg. of the dye (dichlorophenol-indophenol) are extracted twice with 25 ml. portions of boiling water, and each extract poured through a filter into a 50 ml. volumetric flask. The solution is cooled and diluted to the mark. This stock solution will keep about 2 weeks. For daily use, 5 ml. are diluted to 100 ml. with water which has been freshly boiled and cooled.

Preparation of Standard Ascorbic Acid Solution.—Sixty mg. of ascorbic acid are weighed and dissolved in 100 ml. of 5 per cent. acetic acid. One ml. of this solution is diluted to 50 ml. with 5 per cent. acetic acid. This dilute standard solution contains 1·2 mg. ascorbic acid in 100 ml.

Folin and Ciocalteau's Phenol Reagent.—This is made by dissolving 100 g. of sodium tungstate, $Na_2WO_4.2H_2O$, and 25 g. of sodium molybdate, $Na_2MoO_4.2H_2O$, in 700 ml. of water, contained in a 1500 ml. flask, adding 50 ml. of phosphoric acid (s.g. 1·75) and 100 ml. of concentrated hydrochloric acid. The flask is connected to a refluxing condenser by means of a ground-glass joint or by using a rubber stopper wrapped in tin foil,† the mixture boiled for 10 hours; then 150 g. of lithium sulphate, 50 ml. of water and a few drops of bromine are added. The boiling is continued without a condenser for 15 minutes, the golden-yellow solution allowed to cool and diluted to 1 litre.

† If tin foil is employed care must be taken to ensure that the solution does not come into contact with the metal.

Chapter IV

PROCEDURES FOR SERUM

TAKING OF BLOOD FOR SERUM

About 10 ml. of blood are drawn (as for plasma) and allowed to flow gently into a clean dry vessel. This is then kept at room temperature, or in water at about 37° C., until the serum has separated. The blood must not be chilled, as this causes haemolysis as well as abnormal plasma/cell distribution of certain ions. The serum is poured from the clot into a centrifuge tube and is finally centrifuged.

SERUM CALCIUM

There are normally present about 10 mg. of calcium in 100 ml. of serum from freshly clotted blood. In hyperparathyroidism and conditions of generalized bone disease this amount may be raised by 2 or 3 mg. and occasionally in severe cases by 5 mg. or even more. In infantile tetany, or in tetany resulting from removal of part or all of the parathyroid glands, the serum calcium is lowered to 7 or 8 mg., and values as low as 6 mg. are sometimes encountered. Those cases of nephritis where an acidosis with raised blood phosphate is present, may also show a lowered serum calcium.

PRINCIPLE

The calcium is precipitated from diluted serum by oxalate, and the washed precipitate of calcium oxalate is titrated in acid solution with standard potassium permanganate. From the equation (see standard solutions) it can be calculated that 1 ml. of 0·01 N-permanganate is equivalent to 0·2 mg. of calcium.

METHOD

Two ml. of clear serum are added to 2 ml. of water in a clean 15 ml. conical centrifuge tube and 2 ml. of saturated (4 per cent.) ammonium oxalate added, with thorough mixing of the liquids. After 30 minutes the mixture is centrifuged, and the supernatant fluid carefully poured off so as not to disturb the precipitate. The tube is allowed to drain for several minutes by standing it, top down, on a piece of filter paper, and then wiping the rim clean of adhering fluid. The precipitate is washed by blowing in 3 ml. of dilute ammonia (2 ml. concentrated ammonia diluted to 100 ml. with distilled water), so that the mat of calcium oxalate at the bottom of the tube is thoroughly stirred up. A further ml. of dilute ammonia is used to wash down the sides of the tube which is centrifuged immediately and drained as before.

The washed precipitate of calcium oxalate is dissolved in 2 ml. of N-sulphuric acid by warming the tube to 70–80° C. in a beaker of warm water. Titration is carried out by adding N/100 potassium permanganate drop-wise from an accurate 2 ml. burette. The tube should be kept warm by immersion in the hot water during the titration. The first drop of permanganate to produce a " persistent " pink colour (for at least 2 minutes) is taken as the end point. A " blank " titration should be carried out with 2 ml. of N-sulphuric acid (with no calcium) and the amount of permanganate used in the blank (usually 1 drop) subtracted from the former titration.

CALCULATION

$$\text{Calcium}^* = \text{ml. } 0{\cdot}01 \text{ N-}KMnO_4 \times 0{\cdot}2 \times \frac{100}{2}$$

$$= \text{ml. } 0{\cdot}01 \text{ N-}KMnO_4 \times 10$$

* mg. per 100 ml. serum.

SERUM POTASSIUM

The serum of normal persons contains 16–20 mg. of potassium per 100 ml. This is a very constant value. There

is a rise in Addison's disease, a small rise during attacks of bronchial asthma and in advanced renal disease, and a fall in familial periodic paralysis during the attacks of paralysis.

PRINCIPLE

The potassium is precipitated as cobalti-nitrite. The precipitate is washed, dissolved in hot water, and an excess of choline hydrochloride and sodium ferrocyanide is added. An emerald-green colour develops immediately, the depth of which is proportional to the amount of cobaltous salt present, and hence to the amount of potassium.

METHOD

This method is adapted from the procedure of Jacobs and Hoffman (1931). 0·5 ml. of serum (or of standard potassium solution) is placed in a conical centrifuge tube graduated at 4 ml., and 1 ml. of filtered Kramer and Tisdall's sodium cobalti-nitrite reagent is added slowly with constant shaking. After 45 minutes 1 ml. of water is added, and the contents are mixed and centrifuged at moderate speed for 15 minutes. The tube is then inverted and drained briefly on filter paper ; 2 ml. of water are added down the sides of the tube without disturbing the precipitate. The tube is again centrifuged for 5 minutes, inverted and thoroughly drained. The precipitate is washed with 5 ml. of 70 per cent. alcohol, and centrifuged and drained. The alcohol is blown into the tube so as to agitate the precipitate. One ml. of water is added and the tube placed in a boiling water-bath until dissolution is complete.

In another graduated centrifuge tube is placed 1 ml. of the standard cobalt solution ($\equiv$ 0·1 mg. potassium) ; 0·5 ml. of choline chloride solution and 0·5 ml. of sodium ferrocyanide solution are added in that order to each tube and the volume made to 4 ml. The colours can be immediately compared in a colorimeter and are stable for several hours. (Orange or red light filter.)

CALCULATION

$$\text{Serum potassium}^* \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} = 0{\cdot}1 \times \dfrac{100}{0{\cdot}5} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 20. \end{cases}$$

* mg. per 100 ml. serum.

SOLUTIONS

Sodium Cobalti-Nitrite Reagent (Kramer and Tisdall, 1921). —Solution A : 25 g. of cobaltous nitrate crystals are dissolved in 50 ml. of water, and to this solution are added 12·5 ml. of glacial acetic acid. Solution B : 120 g. of sodium nitrite (potassium free) are dissolved in 180 ml. of water, giving a total volume of about 220 ml. To the whole of A is added 210 ml. of B. Nitric oxide is evolved. Air is drawn through the solution till all the gas has passed off. The reagent thus prepared is best kept in the ice-chest and should be filtered each time before use ; it keeps for a month.

Standard Potassium Solution.—0·2228 g. of potassium sulphate (K_2SO_4) is dissolved in 500 ml. of water, giving a solution equivalent to 0·2 mg. K per ml. (≡ 20 mg. potassium per 100 ml.).

Standard Cobalt Solution (≡ 0·1 mg. potassium per ml.).— 0·506 g. of cobalt ammonium sulphate is dissolved in a litre of water. Any cobalt salt may be used but this is the easiest to weigh. The solution should be standardized by the above method against the standard potassium solution.

Choline Chloride.—1 g. per 100 ml.

Sodium Ferrocyanide.—2 g. per 100 ml.

Because there are only 16–20 mg. potassium per 100 ml. of serum, and blood corpuscles contain about 300 mg. per 100 ml. there must obviously be no haemolysis. Also, the serum must be separated from the clot soon after the blood is taken ; otherwise potassium will diffuse out into the serum. Separation of serum 1–2 hours after taking the blood is sufficient.

Chapter V

PROCEDURES FOR CEREBRO-SPINAL FLUID

The chemical determinations most frequently of value in the examination of cerebro-spinal fluid are those of protein, chlorides, sugar, calcium and urea. Lange's colloidal gold reaction is also of importance.

Globulin tests in normal fluid are negative. Total protein may be increased in many pathological conditions. Chlorides are lowered characteristically in meningitis, especially tubercular meningitis. Sugar is also lowered in meningitis. Calcium may be lowered in tetany, while the urea value closely parallels the level of blood urea.

Table 6.—*Composition of C.S.F.*

Constituent	Normal range (per 100 ml.)	Clinical conditions in which high values (unless otherwise stated) are found
Urea . . .	15–30 mg.	Increased in nitrogen retention.
Creatinine . .	0·7–1·5 mg.	,, ,, ,, ,,
Sugar . . .	60–100 mg.	Diabetes. Reduced in acute suppurative meningitis.
Chlorides (as NaCl)	700–740 mg.	Nephritis. Decreased in meningitis, particularly tuberculous meningitis.
Proteins (total) .	20–40 mg.	Meningitis. Syphilitic conditions. Froin's Syndrome.
Globulin { Pandy .	reactions	
Globulin { Nonne-Apelt	negative.	...
CO_2-combining power	55–65 ml.	

PRINCIPLES OF METHODS

Total protein is determined by the sulphosalicylic acid method, with the use of permanent standards. The reactions of Nonne-Apelt and Pandy are used to test for globulin. Nonne-Apelt's test depends on the precipitation of globulin by

half-saturation with ammonium sulphate. Pandy's reagent is a saturated aqueous solution of phenol.

For chlorides, Mohr's method is employed. This depends on the titration of the fluid with silver nitrate, until all the chloride is precipitated as silver chloride. The first excess of silver nitrate gives, with potassium chromate added as indicator, a reddish-brown precipitate of silver chromate.

Urea, creatinine, sugar, CO_2-combining power and calcium are estimated exactly as in the case of blood.

METHODS

Total Protein. 1 ml. of fluid in a test-tube is treated with 3 ml. of aqueous sulphosalicylic acid (3 g. per 100 ml.). After 5 minutes the turbidity is compared against the permanent standards (p. 151) used for albumin in urine. For values above 90 mg. per 100 ml., the fluid must be suitably diluted before treatment.

Globulin

(1) *Nonne-Apelt's Test.* One ml. of saturated ammonium sulphate in a small tube is carefully layered with about 1 ml. of cerebro-spinal fluid. A white ring at the junction of the liquids indicates globulin.

(2) *Pandy's Test.* About 0·5 ml. of fluid is treated with 1 drop of Pandy's reagent (about 7 g. of phenol per 100 ml. of distilled water). A turbidity or precipitate indicates globulin.

Chlorides. One ml. of cerebro-spinal fluid is treated with 5 ml. of water and 2 drops of potassium chromate solution (10 g. per 100 ml.). The mixture is titrated with M/58·5 silver nitrate solution (containing 2·906 g. of silver nitrate crystals in 1 litre of solution in distilled water), until a permanent reddish-brown colour is produced.

NOTE.—The silver nitrate solution should be kept in the dark in a brown bottle and should be frequently checked against a standard sodium chloride solution (500 mg. per 100 ml.).

CALCULATION

1 ml. of $\frac{M}{58 \cdot 5}$ silver nitrate ≡ 1 mg. of sodium chloride.

Hence,

C.S.F. chloride * = ml. of silver nitrate used × 100.

* mg. NaCl per 100 ml.

The adsorption indicator method (p. 46) may also be used for C.S.F. chloride.

LANGE'S COLLOIDAL GOLD REACTION

PRINCIPLE

This reaction depends on the fact that although normal cerebro-spinal fluid has no action on a particular colloidal gold solution, fluid from cases of syphilis, disseminated sclerosis, or meningitis may cause various degrees of precipitation of the gold at different dilutions of the C.S.F., which are fairly characteristic for each disease.

Typical responses are :—

Luetic . . .	. 0 1 3 4 3 2 1 0 0 0
Paretic . .	. 5 5 5 4 3 2 1 0 0 0
Meningitic . .	. 0 0 1 1 2 3 2 2 1 0

These figures serve to indicate the degrees of precipitation in tubes 1–10 in that order (see below).

A meningitic type of curve is found in all forms of coccal meningitis and tuberculous meningitis. A paretic type of curve is found in G.P.I., in tabes, in disseminated sclerosis and rarely in encephalitis lethargica. When a paretic curve occurs in association with a positive W.R. and is unaffected by antispecific treatment it is symptomatic of G.P.I. rather than tabes : when it occurs with a negative W.R. it is strongly suggestive of disseminated sclerosis. A luetic type of curve occurs in all forms of cerebral syphilis. It may also be found in disseminated sclerosis and is more common in encephalitis lethargica than the paretic type.

METHOD

Ten small perfectly clean test-tubes (1·0 × 7·5 cm.) are employed. In the first tube is placed 0·9 ml. of sodium chloride solution (0·4 g. per 100 ml. in re-distilled water); and 0·5 ml. of the chloride solution is placed in each of the other nine tubes. To the first tube is added 0·1 ml. of the cerebro-spinal fluid. 0·5 ml. of the mixed contents of this tube are placed in tube 2; 0·5 ml. of this mixture from 2 is transferred to tube 3, and so on, until tube 10 is reached; from this 0·5 ml. is discarded. The dilutions of fluid are then 1 : 10; 1 : 20; 1 : 40; . . . 1 : 5120. 2·5 ml. of colloidal gold (see below) are added to each tube, and the stoppered tubes left for 16–24 hours. At the end of this time precipitation is gauged as follows :—

Complete precipitation (clear supernatant fluid)	is called 5
Partial precipitation (slightly cloudy, light blue supernatant fluid)	,, ,, 4
Deep blue colour	,, ,, 3
Lilac to purple colour	,, ,, 2
Lilac colour	,, ,, 1
Unchanged red colour	,, ,, 0

SOLUTION

Colloidal Gold Solution. (*a*) *Oxalate Method.*—About 50 ml. of re-distilled water are heated rapidly to boiling in a hard glass flask. This water is discarded, and the flask is washed out with fresh cold re-distilled water. One hundred ml. of the water and 1 ml. of the potassium oxalate solution are then heated in the flask almost to boiling-point. The gold chloride solution is then added drop by drop from a 1 ml. pipette to the continuously agitated contents of the flask until the mixture assumes a bright red colour, when it is removed from the flame. The rest of the 1 ml. of gold solution is then added more rapidly. The colloidal gold so formed should be bright cherry-red in colour and should show practically no cloudiness or fluorescence. It will keep for a considerable period.

This preparation requires considerable practice. It is essential that all glassware used be perfectly clean, and that for all solutions, water twice distilled from glass should be used. A chromic acid mixture (20 g. of potassium dichromate dissolved in a minimum amount of water and the volume made to 1 litre with concentrated sulphuric acid) may be used for cleaning glass apparatus, which should be stored in the mixture when not in use.

The solutions required are potassium oxalate (1 g. per 100 ml.) and gold sodium chloride (1 g. of the salt $NaAuCl_4.3H_2O$ per 100 ml. of water).

(*b*) *Citrate Method.*—In a litre flask are placed 675 ml. water twice distilled from glass. The flask is closed with a ground-in reflux condenser and brought to the boil. Seven ml. of 1 per cent. gold chloride solution are added, followed by 17·5 ml. of 1 per cent. trisodium citrate solution. The boiling is continued for 15 min. The flame is removed and the mixture allowed to cool. The resulting solution is a clear sparkling red colour.

To standardize this solution, four 50 ml. samples are taken and 0·15 ml. of N/10 hydrochloric acid, 0·175 ml., 0·2 ml., and 0·225 ml. are added. These are put up against a known paretic C.S.F. The mixture is selected which gives the strongest reaction with a paretic fluid, yet does not give a reaction greater than 0110000000 with a normal fluid. The corresponding quantity of acid is added to the main bulk of solution, thoroughly agitating the mixture during the addition.

If a paretic C.S.F. is not available, a solution of 0·8 per cent. washed haemolyzed sheep cells, used in the test like a C.S.F., will give a paretic curve.

Chapter VI

PROCEDURES FOR FAECES

BLOOD IN FAECES

The recognition of small amounts of blood in faeces (" occult blood ") indicates the presence of a haemorrhage somewhere in the intestinal tract. Bleeding from the mouth and throat, and contamination of the faeces by menstrual or haemorrhoidal bloods should, of course, be ruled out. (In the latter case the undecomposed blood may be detected macroscopically—usually as superficial patches on the stool).

" Occult blood " occurs fairly frequently in gastro-intestinal cancer and in gastric and duodenal ulcer. Its detection is therefore of considerable value as an aid to diagnosis. Repeated tests are advisable as bleeding may be intermittent and a single negative result is not necessarily significant. A single positive test, or a series of positive tests, on a properly controlled patient, will usually be found to be significant of the presence of some type of ulcer.

Certain of the degradation products of haemoglobin formed during the digestion of meat will give a positive test if they are present in the faeces. Chlorophyll has been alleged to do likewise. It is consequently essential to maintain the patient on a meat-free and green vegetable-free diet for at least three days before making the examination.

PRINCIPLE

The chemical tests for blood depend on the fact that the active grouping involving iron in haemoglobin transfers oxygen from hydrogen peroxide to certain oxidizable substances (benzidine, orthotolidin, gum guaiacum and pyramidone are the most common) to give coloured substances.

Benzidine Reaction

(*a*) A thin faecal suspension is made by shaking a small amount of faeces (about as big as a pea) with 5 ml. of water in a test-tube. The mixture is boiled to inactivate enzymes. (These, as well as blood, may give positive results, but unlike the active grouping of haemoglobin, they are destroyed by heat.) The extract is cooled; about 1 ml. of it, in another test-tube, is treated with 2 ml. of benzidine solution and 1 ml. of hydrogen peroxide solution. If the test is positive a blue colour develops at once.

(*b*) A little faeces is smeared on a glass slide, which is then placed on a boiling water-bath for 5 minutes. A mixture of benzidine and hydrogen peroxide solution (2 : 1) is dropped on the smear. A blue colour is indicative of a positive test.

Gum Guaiacum Reaction

One ml. of a boiled and cooled suspension of faeces, made as above, is mixed in a test-tube with 1 ml. of an alcoholic solution of guaiacum resin. 1 ml. of hydrogen peroxide is then added and the tube is well shaken, with further additions of alcohol to dissolve the precipitated resin. A blue colour indicates a positive test. This test may be modified, as above, for use with slides: in this case a clear mixture (approximately 2 : 1) of alcoholic resin and hydrogen peroxide is dropped on the dried faecal smear.

SOLUTIONS

Benzidine Solution.—Approximately 3 g. per 100 ml. made by shaking 3 g. of benzidine in cold glacial acetic acid (100 ml.) until solution is complete.

Hydrogen Peroxide Solution.—10 vols. (3 per cent.).

Gum Guaiacum in Alcohol ("Tincture of Guaiac").—Approximately 1 g. of resin is dissolved in 100 ml. of industrial spirit. The solution must be freshly prepared; it will keep for about a month.

FAT IN FAECES

Normal faeces contain about 20–25 per cent. of mixed fats (dry weight). These consist of a small amount of neutral fat, 1–2 per cent.; free fatty acids, 9–13 per cent; and fatty acids present as soap (e.g., sodium salt of fatty acid), 10–15 per cent.

In clinical conditions in which there is either deficiency of digestion or of absorption, the amount of total fat may be markedly increased. Thus in pancreatic insufficiency the total fat may constitute 60–80 per cent. of the dry weight of the faeces. Most of the increase is in the neutral fat and may be due to insufficient pancreatic secretion. There is also an increase of fat in cases of deficient bile secretion such as obstructive jaundice. The increase here is mainly due to fatty acid. The hydrolysis of the fats is normal, but they are improperly absorbed, apparently due to a lack of sufficient bile to bring about proper emulsification.

PRINCIPLE

The fats are extracted from the dried faeces by means of an anhydrous solvent (ether) and the weight of the material extracted is ascertained by weighing the residue obtained when the extract is evaporated to dryness. The neutral fat and the free fatty acid of the faeces are soluble in ether and can be extracted directly from the faeces, but it is first necessary to release the fatty acids combined as soap by treatment with hydrochloric acid, before they can be extracted. Free fatty acid is determined by alkali titration.

METHOD

The faeces are prepared for analysis by drying the sample in a porcelain dish on a hot water bath, and then transferring to a vacuum desiccator over calcium chloride. The dry specimen is carefully powdered and thoroughly mixed in a mortar.

A. Total Fat. The faeces are treated with hydrochloric acid to liberate fatty acid present as soap and the whole of the fats extracted with ether. 0·5 g. of the faeces is trans-

ferred to a 100 ml. glass stoppered cylinder. 10 ml. of water and 3 ml. conc. hydrochloric acid are added. The cylinder is placed in a hot water bath for 10 minutes.* After cooling to room temperature 50 ml. of ether are added and the contents thoroughly mixed by inverting the tube 60–80 times. By this means all the fat of the faeces should pass into the ether. The cylinder is left stoppered in a vertical position until the ether layer is completely separated. Twenty-five ml. of the ether extract are now removed and are placed in a weighed evaporating basin. The ether is removed by warming the dish on a water-bath or a hot plate and then placing in a vacuum desiccator containing a few lumps of paraffin wax in a beaker. The increase in weight in the dish represents the fat content of half the total ether extract and hence of 0·25 g. of dried faeces. The result is expressed as g. total fat per 100 g. dried faeces.

B. Neutral Fat plus Free Fatty Acids (unsoaped fat). An ether extraction of the faeces which have not been treated with hydrochloric acid will dissolve out the neutral fat and the free fatty acid without removing any of the fat present as soap. 0·5 g. of dried faeces is transferred to another 100 ml. glass-stoppered cylinder and 10 ml. of water are added without the hydrochloric acid used in the case of total fat. After warming for 10 minutes as in " A " the cylinder is cooled and 50 ml. of ether added. The ether soluble fat is extracted as before by thorough mixing of the contents of the tube. Twenty-five ml. of the extract are removed to a weighed dish and from the weight of the residue the g. of neutral fat plus free fatty acid in 0·25 g. of dried faeces is obtained.

C. Free Fatty Acid. The free fatty acids in the residue from (B) are determined by titration with N/10 sodium hydroxide in alcohol. The residue is dissolved in 10–20 ml. of absolute alcohol and a few drops of phenolphthalein added. The alcoholic sodium hydroxide is run in from a burette until the production of the first permanent pink colour. The

* Care must be taken to warm the cylinder gradually, otherwise it will crack.

result is calculated on the arbitrary assumption that all the free fatty acid present is stearic acid (molecular weight 284).

1 ml. N/10 sodium hydroxide ≡ 0·0284 g. free stearic acid.

D. Fatty Acid present as Soap. By subtracting the weight of the residue of the ether extract of neutral fat and fatty acids from that of the extract of total fat the weight of the fatty acid present as soap may be obtained; i.e., D = A − B.

E. Neutral Fat. By subtracting the amount of free fatty acid (obtained by titration) from the weight of (B) (the neutral fat plus free fatty acid) the amount of neutral fat can be obtained, i.e., E = B − C.

SOLUTION

N/10 *Sodium Hydroxide in Alcohol.*—10 ml. of N-sodium hydroxide are diluted to 100 ml. with absolute alcohol.

Chapter VII

URINE

Approximate Average Daily Composition of Human Urine

Nitrogenous Constituents—

Urea	30 g.	(as N, 14 g.)
Ammonia	0·8 g.	(as N, 0·7 g.)
Creatinine	1·5 g.	(as N, 0·56 g.)
Hippuric Acid	0·5 g.	(as N, 0·04 g.)
Amino-Acids		(as N, 1 g.)
Uric Acid	0·4 g.	(as N, 0·17 g.)

Urochrome and other pigments.

Sulphur-containing Constituents—

Inorganic sulphates (as H_2SO_4), 1·8 g.
Ethereal sulphates, e.g., indican, 0·3 g.
" Neutral " sulphur compounds, e.g., NaCNS, 0·3 g.

Other Constituents—

Organic

Oxalic acid	0·02 g.

Carbonic acid
Aromatic oxyacids, e.g., *p*-hydroxyphenylacetic.

Inorganic

Phosphate (as P)	1·7 g.
Chloride (as NaCl)	12 g.
,, (as Cl)	7·3 g.
Sodium	10 g.
Potassium	2·5 g.
Calcium	0·2 g.
Magnesium	0·2 g.
Water	1400 ml.

PROCEDURES FOR URINE

SUGAR

The most satisfactory solution for use in the clinical detection of sugar in urine is that devised by Benedict. It will keep indefinitely and is not affected by uric acid or creatinine which reduce some of the other reagents.

Benedict's Test. To 5 ml. of the reagent in a test-tube are added 8 drops of the urine to be tested. The mixture is boiled vigorously for 2 minutes in a bare flame, or 5 minutes in a boiling water-bath. In the absence of glucose the solution will remain perfectly clear or at most show only a faint turbidity of flocculent precipitate of phosphate. If clear print, in a good light, cannot be seen through the turbidity, the test may be considered positive. As the amount of sugar increases, the appearance of the precipitate varies from green to yellow and finally to orange with proportionate discharge of the original blue colour. (If much albumin is present in the urine it must be removed by precipitation with heat and acetic acid, followed by filtration.) The sugars, glucose, lactose, fructose and pentoses may be found in urine. Of these, glucose and lactose are the most common.

Distinction between Glucose and Lactose. Glucose and lactose both reduce Benedict's solutions. Lactose sometimes occurs in urine of pregnancy and lactation, and it is important to distinguish it from glucose. The following tests are used :

(*a*) *Fermentation Test.* A large test-tube is filled with urine, and a smaller tube, also filled with urine and containing a little yeast, is inverted in it. By closing the larger tube and inverting it carefully, the smaller tube is freed from air bubbles and sinks, in the inverted position, to the bottom of the large tube. In the presence of glucose fermentation will occur, and after a few hours the small tube will contain bubbles of carbon dioxide, the accumulation of which may bring it to the surface. If lactose alone is present no gas is produced.

(*b*) *Osazone Test.* To 5 ml. of the urine in a wide test-tube are added 0·2 g. of phenylhydrazine hydrochloride, 0·4 g. of sodium acetate crystals and 1 drop of glacial acetic acid. The mixture is heated for 45 minutes in a boiling water-bath, and is then filtered and allowed to cool slowly. Glucose and lactose each form characteristic yellow osazones, distinguishable by microscopic examination. Lactosazone forms globules or clusters of fine crystals resembling fluffy yellow balls. Glucosazone gives lath-shaped crystals.

ESTIMATION OF GLUCOSE

In diabetes mellitus it is frequently useful to determine the amount of sugar excreted in the urine. The dosage of insulin may be gauged by the daily excretion of sugar, the patient being under dietary control. The efficacy of the treatment may be most accurately determined by means of the estimation of the fasting blood sugar in conjunction with the above, but when facilities for the determination of blood sugar are not available it is possible to handle diabetes with reasonable success by the use of urine sugar estimations alone.

Meyer's Modification of Benedict's Method

The method affords a rapid and simple means for the estimation of sugar in urine.

Five ml. of Benedict's quantitative reagent together with 1 to 2 g. of sodium carbonate are placed in a large test-tube. About 5 ml. of distilled water are added to the mixture. The solution is brought to the boil over a small flame. The urine is added drop by drop from a 1 ml. graduated pipette. A few seconds' boiling is allowed between each addition of urine. The end point is recognized by the disappearance of the blue and green colour. Five ml. of Benedict's solution are completely reduced by 10 mg. of glucose, 11·6 mg. of galactose, or 13·4 mg. of lactose. Fresh batches of Benedict's reagent should be tested with 1 g./100 ml. solutions of pure glucose and lactose (also with galactose if the reagent is to be used for the galactose excretion test).

SOLUTIONS

Composition of Benedict's Reagents

Qualitative Reagent. Sodium citrate (173 g.) and anhydrous sodium carbonate (100 g.) are dissolved together in about 600 ml. of water. The solution, filtered if necessary, is poured into a 1 litre volumetric flask. A solution of copper sulphate (17·3 g.) in 100 ml. of water is added slowly with constant shaking. The volume is then made to 1 litre.

Quantitative Reagent. Sodium citrate (200 g.), anhydrous sodium carbonate (100 g.) and potassium thiocyanate (125 g.) are dissolved together with the aid of heat in about 600 ml. of water, and the solution, filtered if necessary, is poured into a 1 litre volumetric flask. A solution of copper sulphate (exactly 18·0 g. in 100 ml. of water) is then added gradually to the well-shaken mixture, followed by 5 ml. of a 5 per cent. solution of potassium ferrocyanide. The volume is made to 1 litre. Five ml. of this solution should be equivalent to 10 mg. of glucose, but a freshly made solution should be standardized against a 1 per cent. glucose solution.

"ACETONE"

The "acetone" bodies in urine include acetoacetic and β-hydroxybutyric acids in addition to acetone.

Rothera's Test

To one-third test-tube of solid ammonium sulphate are added not more than 5 ml. of urine and 8–10 drops of fresh sodium nitroprusside solution, or a few granules of the powdered solid. After the addition of 1–2 ml. of strong ammonia the tube is shaken well and allowed to stand. A permanganate colour indicates the presence of acetoacetic acid. If the quantity is small the colour may develop slowly.

This test detects 1 part of acetoacetic acid in 100,000 parts of urine.

Gerhardt's Test

To 5 ml. of urine is added, drop by drop, a solution of ferric chloride (3 per cent.). White ferric phosphate is precipitated. More ferric chloride is added and the solution is filtered. A brown or purplish colour is obtained depending on the amount of acetoacetic acid present. The urine tested must be free from salicylic acid and salicylates and the compounds excreted after administration of aspirin, antipyrin, etc., all of which give a very similar reaction to acetoacetic acid, but never give the sodium nitroprusside reaction.

This test detects 1 part of acetoacetic acid in 7,000 parts of urine. The colour, if due to acetoacetic, is discharged on heating; if due to salicylates, it persists.

NOTES.—When Rothera's test is found positive, Gerhardt's test should always be done in order that a rough estimate of the degree of ketosis may be made. When Rothera's test is negative no acetone bodies are present and other tests become unnecessary.

The nitroprussic reaction is a test for acetone, but acetoacetic acid decomposes so readily into acetone that it also gives the test. The ferric chloride test for acetoacetic acid is not given by acetone.

SPECIFIC GRAVITY OF URINE

The urinometer is placed in the urine in the urinometer cylinder or a 50 ml. graduate. The urinometer should float freely in the urine and not touch the side of the vessel. In reading the urinometer the eyes should be on a level with the top of the meniscus of the urine both at the front and back of the cylinder. On the stem of the urinometer the number just visible above the surface of the urine is noted. This is the specific gravity to which 1000 is to be added.

ALBUMIN

Protein (in significant amount) in urine may be albumin and globulin (derived from the blood plasma) or "Bence-Jones protein."

Albumin and Globulin. These proteins are found in the urine (albumin greatly predominating in amount) both in " normal " and pathological conditions. " Normal " albuminuria occurs occasionally in youthful subjects and is generally increased in degree after exercise and decreased during rest. Thus, a morning specimen of urine may show a negative test for albumin, whereas one taken after exercise may contain considerable quantities of protein. This so-called " orthostatic albuminuria " is not generally associated with renal or cardiac deficiency and frequently disappears in later life. The chief pathological conditions associated with albumin in the urine are the renal diseases such as the various forms of nephritis and nephrosis. The amount of protein voided is variable and the protein is mainly albumin.

" Bence-Jones Protein " is excreted in many cases of multiple myelomatosis and in other less well-defined conditions. Small amounts of protein excreted with blood, pus, or bile (without gross proteinuria) often give a positive test for " protein." In these cases, the amounts detected may be little more than the minimum detectable amount, namely about 10 mg. per 100 ml. of urine.

Heat and Acetic Acid Test

About 10 ml. of the urine (filtered if cloudy) are placed in a test-tube. The tube is warmed in a small flame at a place about 3 inches above the bottom. A cloud will probably form at this place ; if this is so, 4–5 drops of dilute (5 per cent.) acetic acid are added and the tube re-heated. If the cloud is due to phosphates it will now vanish ; if albumin is present the cloud remains and may increase.

If " Bence-Jones Protein " is present, the cloud originally formed will vanish on cooling, reappear when heated to 60–70° C., and then disappear again at boiling temperature, where any albumin may be removed by filtration.

Sulphosalicylic Acid Test

One ml. of clear urine (filtered if necessary) in a test-tube is mixed with 3 ml. of sulphosalicylic acid solution (3 g. per 100 ml.). A precipitate indicates the presence of albumin.

Quantitative Estimation of Albumin

The urine and sulphosalicylic acid are mixed in the proportions named above in a small tube of standard size. After 5 minutes, the turbidity is compared with that in the set of gelatin permanent standards (King and Haslewood 1936; see page 151), and read as mg. of albumin per 100 ml. of urine. If the amount is greater than 100 mg. albumin per 100 ml. the urine is suitably diluted and a fresh estimation carried out.

NOTE.—The above tests will detect albumin in urine in amounts as little as 10 mg. per 100 ml. Unlike the nitric acid tests they do not give " false positive " results.

BLOOD

Using boiled and cooled urine the benzidine or guaiacum test is carried out, as described on page 75. Urine containing blood frequently gives a positive test for albumin.

UREA

Estimations of the amount of urea and of ammonia in the urine are valuable in a number of tests of renal function. The amount of urea in a single specimen may be from 0·02–4·00 g. per 100 ml., while the quantity of ammonia is usually small. The 24-hourly excretion is, approximately, 30 g. of urea and 0·8 g. of ammonia. In certain conditions the ammonia content of the urine may be greatly raised (e.g., in diabetic acidosis).

PRINCIPLES OF METHODS

(*a*) The urea is converted to ammonia with urease and the ammonia nitrogen estimated by Nesslerization.

(*b*) The urea is converted to nitrogen by sodium hypobromite and the nitrogen measured manometrically.

Method (*a*) is used for the accurate estimation of urea and ammonia, while method (*b*) is particularly well adapted for use in renal function tests where a rapid and fairly accurate measure of the amount of urea + ammonia is required.

UREASE-NESSLER METHOD FOR UREA AND AMMONIA

PRINCIPLE

The urea is converted into ammonia with urease and the ammonia formed is estimated colorimetrically after Nesslerization. The preformed ammonia is determined in another sample and the urine urea content calculated by subtracting the ammonia content from the urea + ammonia content.

METHOD

A. Urea and Ammonia. One ml. of urine is pipetted into a 50 ml. volumetric flask together with about 25 ml. of water. A " knife-point " of Jack Bean meal is added and the flask stoppered, well-shaken and incubated at 37° C. for 20 minutes. Two ml. of 10 per cent. zinc sulphate and 2 ml. of N/2 sodium hydroxide are added and the volume of liquid is made to 50 ml. The well-mixed contents are allowed to stand 5 minutes and are then filtered.

One ml. of the filtrate (≡ 0·02 ml. of urine) is pipetted into a 50 ml. volumetric flask. Into two other 50 ml. volumetric flasks are pipetted 10 ml. and 20 ml. respectively of standard ammonium chloride solution (0·01 mg. N per ml.). Water is added to each flask to about 40 ml., and after the addition of 5 ml. of Nessler's reagent more water is added to the 50 ml. mark. The test solution is then read in the colorimeter against the standard which is nearer in colour to it. The use of a violet light filter facilitates the comparison.

CALCULATION

(1) Where the standard is 10 ml. of ammonium chloride,

$$\text{Urine urea + ammonia}^{*} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}1 \times \frac{100}{0{\cdot}02}$$

(2) Where the standard is 20 ml. of ammonium chloride,

$$\text{Urine urea + ammonia}^{*} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}2 \times \frac{100}{0{\cdot}02}$$

* mg. N per 100 ml.

To express the results as urea multiply by 2·14.

B. Ammonia. One ml. of the urine is placed in a 50 ml. volumetric flask together with about 25 ml. of water; 2 ml. of sodium hydroxide and 2 ml. of zinc sulphate are added and, after diluting to the mark, the contents are filtered. Ten ml. of the filtrate (≡ 0·2 ml. of urine) are pipetted into another 50 ml. flask, water is added to about 40 ml. and after the addition of 5 ml. of Nessler's reagent and dilution to the mark, the colour is read in a colorimeter in the same way and using the same standards as described for urine urea + ammonia.

CALCULATION

(1) When the standard is 10 ml. of ammonium chloride,

$$\text{Urine ammonia}^* = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}1 \times \frac{100}{0{\cdot}2}$$

(2) When the standard is 20 ml. of ammonium chloride,

$$\text{Urine ammonia}^* = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 0{\cdot}2 \times \frac{100}{0{\cdot}2}$$

* mg. N per 100 ml.

NOTES.—If urea only is required this is of course calculated by subtracting the ammonia value from urea + ammonia figure.

For the purpose of the urea clearance test the urea + ammonia figure is used since, as Van Slyke has pointed out, this result is a better index of the rate of urea excretion.

Hypobromite Method

The apparatus sketched is used. The 50 ml. burette A is clamped vertically and its top end is closed with a doubly bored rubber bung. Through one of the holes in this bung passes a short glass tube closed by a piece of pressure tubing and a clip B. Through the other hole passes a short tube connected with pressure tubing to the small bottle C, which is closed by a rubber bung. The other end of the burette is connected to the reservoir D, which is filled with water.

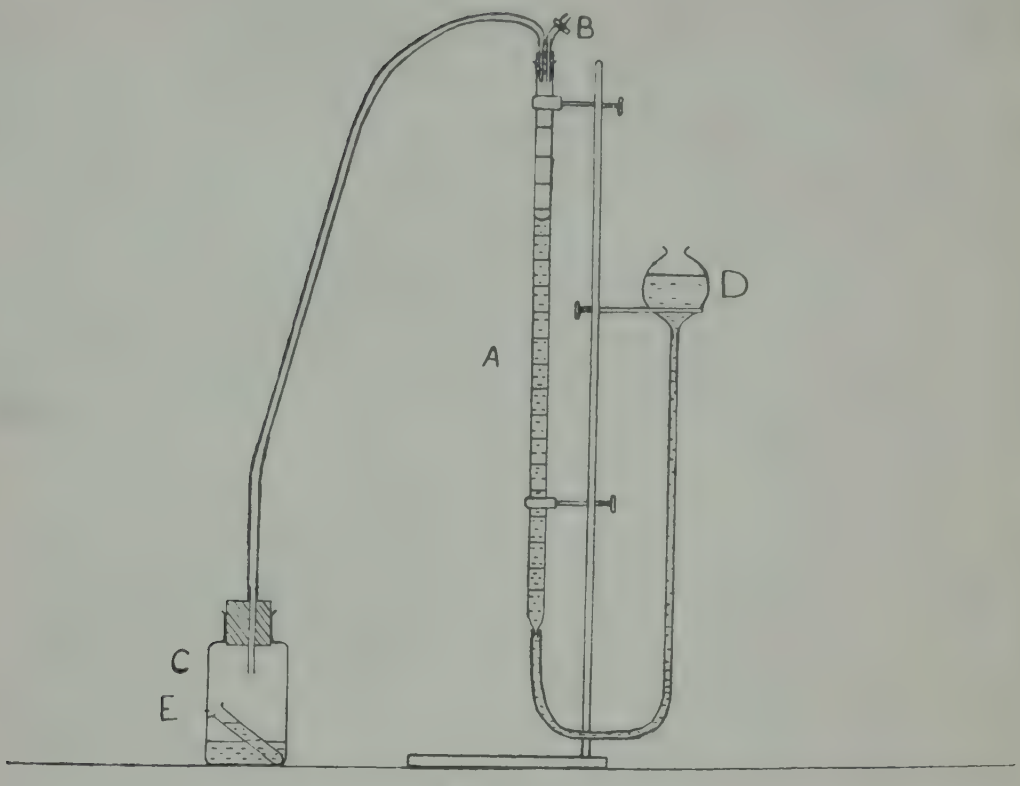


FIG. 3.

METHOD

The estimation is carried out as follows : 10 ml. of sodium hypobromite solution are placed in the bottle C, and 2 ml. of urine in a small tube E, which rests in C. C is then tightly stoppered, and placed in a large beaker of water for 5 minutes. The clip B is opened and the water level is adjusted to zero by moving D. B is then closed, and the contents of E mixed with the hypobromite by tipping. C is now well shaken until reaction ceases, when C is placed in the beaker for a further 5 minutes. The level of water in the burette is now read with the level of water in D at the same height. This reading is the volume of nitrogen evolved, under the conditions, from 2 ml. of urine.

CALCULATION

The theoretical reaction is as follows :

$$\underset{\text{urea, 60 g.}}{CH_4N_2O} + 3NaOBr + 2NaOH \longrightarrow \underset{(22{\cdot}4\ \text{l.})}{N_2} + 3NaBr + Na_2CO_3 + 3H_2O.$$

Hence, 60 g. urea ≡ 22·4 l. of nitrogen at N.T.P.
∴ 1 g. „ ≡ 373 ml. „ „ „ „

In fact, 357 ml. of nitrogen are yielded on the average by 1 g. of urea, and it is not necessary to correct for temperature and pressure.

Hence, $357 \text{ ml. } N_2 = 1 \text{ g. Urea}$

$$\therefore \quad \text{Urea in urine}^* = \frac{\text{ml. } N_2}{357} \times \frac{100}{2}$$

$$= \frac{\text{ml. } N_2}{7 \cdot 14} = \text{ml. } N_2 \times 0 \cdot 14$$

* g. per 100 ml.

Reagent.—Sodium hypobromite (prepared fresh, daily). 2·5 ml. of bromine are carefully added to 25 ml. of sodium hydroxide (40 g. per 100 ml.).

TOTAL NITROGEN OF URINE

Total nitrogen of urine consists of the nitrogen-containing compounds : ammonium salts, urea, uric acid, creatinine and small amounts of other substances, some of them still unidentified. The total nitrogen excreted by a normal adult individual is about 14 g. per day. This amount may be depressed in conditions involving kidney damage.

PRINCIPLE

The Kjeldahl Method

The organic substances of the urine are destroyed by digestion with concentrated sulphuric acid and all the nitrogen present is converted into ammonium sulphate. By the addition of excess of alkali, ammonia is liberated and is quantitatively distilled off into a measured amount of standard acid. The amount of acid neutralized by the ammonia gives the measure of the amount of nitrogen present in the sample of urine used.

METHOD

One ml. of urine is accurately measured into one of the small digestion flasks of the micro-Kjeldahl outfit. Two ml. of concentrated sulphuric acid are added and a small amount of copper sulphate, potassium sulphate and selenium dioxide. The mixture is heated on the special digestion apparatus. It gradually turns black as the organic material is charred, then becomes light brown, and finally a clear blue. The flask

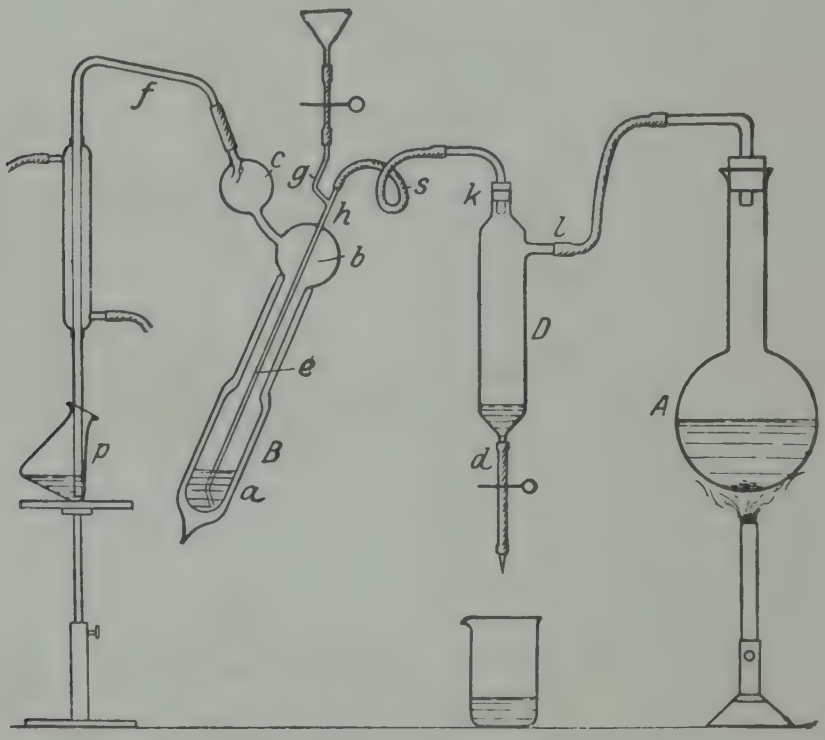


FIG. 4.—Parnas & Wagner's improved micro-Kjeldahl apparatus (Pregl).

is heated for 2 hours, cooled to room temperature and the contents diluted with distilled water. The solution is quantitatively transferred to the distilling apparatus (fig. 4) by pouring it through the small funnel attached and following by successive washings of the flask with small amounts of distilled water. Ten ml. of 0·1 N-sulphuric acid are now added to a 100 ml. conical flask. Two drops of methyl red indicator are added and the flask placed on the stand beneath the condenser tube of the distillation apparatus. The height of the flask is adjusted so that the bottom of the distillation

tube dips below the surface of the liquid. Ten ml. of concentrated alkali (40 per cent. NaOH) are now added to the distillation apparatus through the small funnel. The ammonia which is liberated is distilled over into the standard acid in the conical flask by bubbling steam through the mixture. A Bunsen burner is placed under the large round bottomed boiling flask which should be about half full of water. Steam is bubbled through the mixture and the distillation continued until the contents of the conical flask are about twice what they originally were. The flask is now lowered until the end of the condenser tube no longer dips into the liquid. The condenser tube is washed down with distilled water into the conical flask, which is now removed and its contents titrated with 0·1 N-sodium hydroxide. The titration figure so obtained is subtracted from the 10 ml. of 0·1 N-sulphuric acid to give the ml. of standard acid which were neutralized by the ammonia.

CALCULATION

ml. 0·1 N-H_2SO_4 neutralized by ammonia $\times$ 1·4
= mg. nitrogen in the 1 ml. of urine.

It is usual to express the nitrogen content of urine in terms of the output per 24 hours.

NOTE.—Determinations of the different nitrogen constituents of urine are made according to procedures which are essentially the same as those used for blood.

CREATININE AND CREATINE

Creatine is methyl-guanidine-acetic acid.

Creatinine is its anhydride. These substances are probably derived from protein.

The excretion of *creatinine* in the normal individual varies according to the muscular weight. The amount eliminated each 24 hours ranges between 0·4 and 1·8 g. in the adult. On a creatine-free diet the output is remarkably constant and very seldom varies except in uncommon clinical conditions.

The excretion of *creatine* in normal adult females is intermittent; and in adult males creatine is very seldom present and only in small quantities. It is increased in the urine in any condition which raises the B.M.R., in the myopathies and in several endocrine disturbances.

PRINCIPLE OF METHOD FOR CREATININE

The Folin method is based upon the production of an orange-red colour by the interaction of creatinine with alkaline sodium picrate. The colours produced are compared in a colorimeter, and the creatinine content of the urine estimated by comparison with a creatinine solution of known strength.

METHOD

A volume (usually 1–3 ml.) of urine containing 0·7 to 1·5 mg. creatinine, is measured with a pipette into a 100 ml. volumetric flask. Into a similar flask is pipetted 1 ml. (≡ 1 mg. of creatinine) of a standard creatinine solution. To each flask are added 4 ml. of N-sodium hydroxide and 20 ml. of saturated picric acid. After 10 minutes each flask is filled to the mark with water and the contents mixed. The colours are compared in a Duboscq colorimeter. (For photoelectric comparison, a "blank" is used for the zero setting.)

CALCULATION

$$\text{Creatinine}^{*} \begin{cases} = \dfrac{\text{reading of standard}}{\text{reading of test}} \times 1{\cdot}0 \times \dfrac{100}{v} \\ = \dfrac{\text{reading of standard}}{\text{reading of test}} \times \dfrac{100}{v} \end{cases}$$

where v is the volume of urine used.

* mg. per 100 ml. of urine.

METHOD FOR CREATINE

Creatine is converted to creatinine by boiling in picric acid solution. Twenty ml. of saturated picric acid solution are pipetted into a flask together with a measured amount of

urine (usually equal to the volume used for the creatinine determination), and about 130 ml. water. The contents are brought to the boil rapidly, and then allowed to boil gently for 1 hour. The flame is raised and the mixture boiled down rapidly to bring the volume of the solution to about 25–30 ml. The flask is cooled, and 4 ml. N-NaOH are added. After 10 minutes the contents of the flask are transferred to a 100 ml. volumetric flask and water is added to the mark. The contents are compared in the colorimeter with the standard coloured solution used for creatinine.

CALCULATION

As for creatinine. The result is total creatinine, i.e., preformed creatinine plus creatine, expressed as creatinine. Thus, the amount of preformed subtracted from the total creatinine gives the creatine content of urine expressed as creatinine.

SOLUTIONS

N-*Sodium Hydroxide.*

Saturated Solution of Picric Acid.

Creatinine "Stock" Standard. (Containing 1 mg. of creatinine per ml.).—1·602 g. of pure creatinine zinc chloride are dissolved in N/10 hydrochloric acid solution, and the volume made up with the N/10 acid to 1 litre. (This is the same "*Stock*" *Standard* as is used in the blood method.)

URIC ACID

Urine contains about 0·4 g. of uric acid per 24-hourly specimen. This amount may be increased in cases of leukaemia, and decreased in gout.

PRINCIPLE

Urine is diluted and treated with Benedict's arsenophosphotungstic acid reagent and then with a sodium cyanide-urea solution. The blue colour produced by reduction, at the

alkaline reaction, of the arseno-phosphotungstic acid, is compared with that obtained under similar conditions from a standard uric acid solution.

METHOD

1 ml. of urine is diluted to 25 ml. in a volumetric flask. Ten ml. ($\equiv 0 \cdot 4$ ml. of urine) of this solution are placed in a 50 ml. volumetric flask, and 10 ml. ($\equiv 0 \cdot 2$ mg. uric acid) of the uric acid urine standard in a similar flask. To each mixture are added 5 ml. of urea-cyanide solution, and 1 ml. of Benedict's uric acid reagent. After 10 minutes, the solutions are made to the mark, mixed and compared in a colorimeter.

CALCULATION.

$$\text{Uric acid } * \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0 \cdot 2 \times \dfrac{100}{0 \cdot 4} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 50 \end{cases}$$

* mg. per 100 ml. of urine.

SOLUTIONS

Benedict's Arseno-phosphotungstic Acid Reagent.—100 g. of sodium tungstate are dissolved in about 600 ml. of water in a litre round-bottomed flask. To the solution are added 50 g. of pure arsenic pentoxide followed by 25 ml. of syrupy phosphoric acid (85 per cent.) and 20 ml. of concentrated hydrochloric acid. This mixture is boiled for 20 minutes, cooled, washed into a 1 l. volumetric flask and diluted to the mark.

Stock Uric Acid Standard (Folin) ($\equiv 1 \cdot 0$ *mg. per ml.*).—(See Blood.)

Uric Acid Urine Standard ($0 \cdot 02$ *mg. per ml.*).—20 ml. of the above standard are diluted with 1 ml. of 40 per cent. formalin and water to 1 litre.

Urea-cyanide Solution.—5 g. of sodium cyanide and 20 g. of urea are dissolved in water, and the volume made to 100 ml.

CHLORIDES

The usual excretion of chloride amounts to about 10 or 12 g. (expressed as sodium chloride) per 24 hours for normal individuals. The amount of this excretion may be altered during certain clinical conditions. It is most markedly depressed in conditions of oedema which are characterized by salt and water retention.

PRINCIPLE

The Volhard procedure for the determination of chloride in solution consists in the precipitation of the chloride with a measured excess of standard silver nitrate in the presence of nitric acid. The excess of silver nitrate over that necessary for complete precipitation is measured by titration with standard ammonium thiocyanate. A small amount of a ferric salt is added as indicator, the first production of a red colour due to the formation of ferric thiocyanate indicating the point at which an excess of ammonium thiocyanate has been added. The ferric salt is included in the silver nitrate solution.

The difference between the volume of the standard silver nitrate used and that of the standard ammonium thiocyanate represents the amount of silver precipitated by the chloride.

METHOD

Ten ml. of urine are added to about 25 ml. of water in a 100 ml. volumetric flask. Twenty ml. of the standard silver nitrate are added and after a few minutes the volume made to the 100 ml. mark. The mixture is filtered, and 50 ml. of the clear liquid transferred to a 250 ml. conical flask for titration. Standard ammonium thiocyanate is run in until the production of the first permanent faint reddish-pink colour.

CALCULATION

The figure representing the thiocyanate titration is subtracted from 10 (ml. of silver nitrate) to give the figure representing the silver nitrate precipitated by chloride.

One ml. standard silver nitrate N/5·85 precipitates 0·01 g. sodium chloride.

∴ (10 − ml. thiocyanate) × 0·01 = g. sodium chloride in 5 ml. of urine (i.e., in half the volume taken).

It is usual to express the chloride content of urine in terms of the output of sodium chloride per 24 hours.

SOLUTIONS

Standard Silver Nitrate N/5·85.—29·060 g. silver nitrate crystals are dissolved in about 400 ml. of water in a litre flask. Forty g. ferric ammonium sulphate (alum) are dissolved in a mixture of 100 ml. of water with 85 ml. of concentrated nitric acid (sp. gr. 1·43). This latter solution is added to the former, mixed, and made to the mark. This standard silver nitrate solution is of such a strength, i.e., N/5·85, that 1 ml. is the equivalent of 0·01 g. sodium chloride (molecular weight of NaCl = 58·5).

Standard Ammonium Thiocyanate.—This solution is prepared in such a manner that 1 ml. of it is equivalent to 1 ml. of the standard silver nitrate solution used. Thirteen g. of ammonium thiocyanate (NH_4SCN) are dissolved in 1 litre of water. This gives an approximately N/5·85 solution. Some of it is titrated from a burette into 10 ml. of the standard silver nitrate solution in order to determine its exact strength. The remaining solution is then suitably diluted to make it exactly N/5·85.

SODIUM

The method of estimating sodium in blood plasma is applied to a sample of the urine from which the phosphate has been precipitated with calcium hydroxide.

To about 10 ml. of urine in a small flask, approximately 0·25 g. of dry powdered $Ca(OH)_2$ are added. The mixture is well shaken and is filtered clear. The filtrate should be protected from the air in a stoppered test-tube, to prevent precipitation of $CaCO_3$ from the action of CO_2 on the excess $Ca(OH)_2$. A sample of the filtrate is used for the zinc uranyl

acetate method for sodium in the same manner as blood plasma.

POTASSIUM

The cobaltinitrite method for blood serum is applied to a sample of the urine from which the ammonia, which forms an insoluble cobaltinitrite like potassium, has been eliminated by ashing and re-solution in dilute acid.

Two ml. of urine are evaporated to dryness in a good quality porcelain, vitreosil or platinum crucible. The residue is ashed at a dull red temperature (about 400° C.) for some hours. Overnight is convenient if an electric muffle is available; otherwise the crucible may be placed inside two porcelain evaporating dishes, one inverted over the other, and heated with a Bunsen burner until all carbonaceous matter has disappeared.

The ash is dissolved in a small amount of 0·1 N-hydrochloric acid, and the solution transferred quantitatively with washings of water to a 10 ml. standard flask, made to volume and mixed. (If the potassium content is likely to be high, the solution of the ash is made to 15 or 20 ml.)

0·5 ml. of this solution (= 0·1 ml. of urine) is used for the potassium determination described for blood serum. The result so obtained is multiplied by 5 to allow for the dilution of 2 ml. of urine to 10 ml.

BILE PIGMENT

Urine containing bile is generally yellowish-green to brown in colour. When shaken it foams readily because of the presence of the bile salts. The foam has a yellowish-green colour. This in itself is a rough test for bile but the more accurate clinical tests depend upon the fact that bilirubin is oxidized by nitric acid to form a series of coloured compounds, biliverdin (green), bilicyanin (blue), choletelin (yellow), etc.

Nitric Acid Test

When a urine containing bile pigment is filtered the bilirubin is largely retained on the filter paper. Twenty to

thirty ml. of urine are filtered and *all* of it allowed to drain through the paper. The filter paper is unfolded and spread on top of the funnel. A yellow colour on the paper almost invariably indicates the presence of bilirubin. A drop of concentrated nitric acid is added to the flat paper. If bile pigments are present, the centre of the drop is coloured red and the fringes blue and green, indicating the presence of different oxidation products of the original yellowish-brown bilirubin.

Hunter's Test

Two ml. of 10 per cent. barium chloride are added to 5 ml. of urine in a centrifuge tube. The precipitate of barium sulphate, on which the bile pigments are quantitatively taken up, is spun down and washed with a few ml. of water. 0·5 ml. of Van den Bergh diazo-reagent, 2 ml. of 95 per cent. alcohol, and 0·3 ml. of sodium phosphate buffer ($Na_2HPO_4.12H_2O$—6 g. per 100 ml. of water) are added with thorough mixing after each addition. A red colour indicates bilirubin. (Hunter, 1930.) (Cf. p. 42.)

UROBILIN AND UROBILINOGEN

The amounts of urobilin which are usually present are too small to impart a colour to the urine of normal persons. Most of the pigment is present as the colourless urobilinogen which readily gives rise to urobilin on oxidation. Urines containing large amounts of urobilin are reddish in colour.

Tests

1. Two drops of N/10 iodine solution are added to 5 ml. of urine followed by 5 ml. of a 10 per cent. suspension of zinc acetate in alcohol. The mixture is allowed to settle and in the clear supernatant a green fluorescence becomes apparent if there is urobilin or urobilinogen present. If a spectroscope is available, the fluid may be examined for the broad absorption band (due to urobilin) at the green-blue junction.

2. 0·5 ml. of glacial acetic acid, followed by 2 ml. of 5 per cent. copper sulphate, are added to 10 ml. of urine. Two ml. of chloroform are added and the mixture thoroughly shaken and allowed to settle. If any urobilin is present it will appear in the chloroform layer, to which it imparts a pink colour. The spectrum may again be examined for absorption at the green-blue.

INDICAN

Only small amounts of indican, 5–20 mg. per day, appear in normal urine. Great increases are found in clinical conditions where there is stagnation and putrefaction of intestinal contents. Likewise decomposition of body proteins due to bacterial action, as in gangrene and putrid pus formation, results in large amounts of indican appearing in the urine.

TEST

To 5 ml. of urine in a test-tube are added 5 ml. of concentrated hydrochloric acid and 2–3 drops of *dilute* sodium hypochlorite solution, or of a freshly made saturated solution of bleaching-powder (a little bleaching-powder shaken in 10 ml. of water in a test-tube at room temperature). 2–3 ml. of chloroform are added and the test-tube closed with the thumb and inverted several times. The amount of indican in a normal urine will give a light blue colour in the chloroform in this test : if indican is present in increased amount, the chloroform will be intensely coloured. The test is therefore " controlled " with a normal urine, tested at the same time.

Care must be taken in the above test not to use a large excess of hypochlorite solution, or the blue-coloured chloroform-soluble product will be oxidized to a colourless substance.

MELANIN

The black melanin pigments do not occur in normal urine. Their occurrence is usually associated with melanotic tumours.

The urine may be clear or somewhat darkened on passing, but on standing it rapidly deepens in colour and may become dark-brown or almost black.

Zeller's Test

When urine is shaken with an equal volume of bromine water a yellow precipitate forms which gradually darkens in colour and finally becomes black.

Ferric Chloride Test

On adding a few drops of ferric chloride to the urine in a test-tube there is produced a grey colour followed by a black precipitate (consisting of ferric phosphate with adhering melanin) which re-dissolves with excess ferric chloride.

DIASTASE

The estimation of urinary diastase is of some importance in diseases of the pancreas, in which values higher than normal are found. The " units " used are those of Wohlgemuth. They are given by the number of ml. of 0·1 per cent. starch solution digested by 1 ml. of urine. Expressed in these terms, the normal values are 6–30 units. In pancreatic abnormalities the value may rise to over 100 units.

PRINCIPLE

Different dilutions of the urine, buffered to *p*H 6·1 with phosphate solution, are incubated with a 0·2 per cent. solution of starch. After incubation at 38° C., iodine is added to each sample, and the first tube in which no colour is produced is taken as giving the dilution at which the starch is just completely digested.

METHOD

One ml. of the urine is added to 4 ml. of the buffer solution (*p*H 6·1), making a 1 : 5 dilution of the urine. Seven small

test-tubes are then placed in a rack and 4 ml. of the buffered urine (of dilution 1 : 5) are placed in tube (1). Two ml. of buffer are added to each of tubes (2) to (7). Two ml. from tube (1) are mixed with the contents of tube (2). Two ml. of this mixture from tube (2) are transferred to tube (3), and so forth until tube (7) is reached, from the final contents of which 2 ml. are discarded. The dilutions are then 1 : 5 in tube (1), 1 : 10 in tube (2), 1 : 20 in tube (3), and so on, to 1 : 320 in tube (7).

One ml. of 0·2 per cent. starch solution is then added to each tube and mixed with the other liquids. The tubes are incubated, by immersion in a water-bath, at 38° C. for 30 minutes. At the end of this time 3 drops of N/50 iodine are added to each tube.

CALCULATION

Let the dilution of the urine in the first tube which shows no blue or mauve colour be $1 : x$

This tube contains :

2 ml. of a $1 : x$ dilution of urine, i.e., $\frac{2}{x}$ ml. of urine.

Therefore $\frac{2}{x}$ ml. of urine contains just sufficient diastase to digest 1 ml. of 0·2 per cent. starch solution ;

that is,

$\frac{2}{x}$ ml. of urine digests 2 ml. of 0·1 per cent. starch solution ;

hence,

1 ml. of urine digests $\frac{2}{2/x} = x$ ml. of 0·1 per cent. starch soln.

Therefore, the number of Wohlgemuth units is equal to x, the dilution factor of the tube in which the digestion of the starch is just complete.

Urinary diastase is usually reported in terms of the output of the enzyme for 24 hours.

SOLUTIONS

Phosphate buffer (*p*H 6·1).—This is made by mixing two solutions, A and B.

Solution A. — 11·876 g. of di-sodium phosphate ($Na_2HPO_4.2H_2O$) are dissolved in freshly boiled distilled water, and the volume made to 1 litre.

Solution B.—9·078 g. of potassium di-hydrogen phosphate are dissolved as above, and the volume made to 1 litre.

These two solutions are kept in bottles lined with paraffin-wax. The phosphate buffer *p*H 6·1, used in the method, is made by mixing 15 ml. of solution A with 85 ml. of solution B.

Starch Solution (0·2 *per cent.*).—0·2 g. of starch is shaken in a test-tube with a little cold water. The paste so formed is washed into about 80 ml. of boiling water in a beaker.

When the solution is cool, the mixture is poured and washed into a measuring flask, and the volume made to 100 ml.

0·1 N-*Iodine.*—This contains 12·7 g. of iodine crystals dissolved in a solution of 20 g. of potassium iodide in a little water. The volume is then made to 1 litre.

N/50 *Iodine.*—Is made by appropriate dilution of the 0·1 N-iodine with water.

ASCORBIC ACID

The 24-hourly excretion of urinary ascorbic acid in normal persons (whose intake is 20–80 mg. per day) is in the neighbourhood of 50 per cent. of the intake. In cases of vitamin C deficiency and in certain infections there may be little or no ascorbic acid in the urine (Harris and Ray, 1935).

COLLECTION OF URINE

Urine is collected during 24 hours. Day specimens are at once analysed for ascorbic acid (or preserved with exactly 10 per cent. by vol. of glacial acetic acid). Night specimens are put as soon as voided into a bottle containing exactly 100 ml. of glacial acetic acid and well mixed with the acid. All urine is analysed as soon as possible.

PRINCIPLE

Ascorbic acid rapidly reduces, in acid solution, the dye 2 : 6-dichlorophenol-indophenol to a colourless substance. Urines containing ascorbic acid also effect this reduction and the amount of dye reduced by the urine is a fairly accurate measure of the quantity of the acid present.

METHOD

An appropriate amount of the dye (usually 0·5 ml. of the solution of 2 : 6-dichlorophenol-indophenol) is measured into a small test tube. A drop of 50 per cent. acetic acid is added and the urine rapidly run in from a 2 ml. burette until the red colour of the dye disappears.

CALCULATION

0·5 ml. of 2 : 6-dichlorophenol-indophenol solution contains 0·04 mg. of dye. This amount is reduced by approximately 0·02 mg. ascorbic acid. For accurate work, the dye solution should be checked against the pure vitamin and should be freshly prepared daily.

The amount of ascorbic acid equivalent to the dye taken is contained in the quantity of urine used to decolourize the dye, whence the amount present in the total sample or in 100 ml. of urine is calculated. Where acetic acid has been added as preservative the volume added must be allowed for in the calculation. Results are expressed in mg. ascorbic acid per 100 ml. of urine or as mg. excreted in 24 hours, according to the information desired and the type of test being conducted.

Example. A sample of urine had a total volume of 720 ml. including 100 ml. of glacial acetic acid.

0·5 ml. of dye solution required 0·82 ml. of the acidified urine.

$\therefore$ 0·82 ml. of the acidified urine contained 0·02 mg. of ascorbic acid

Hence 720 ml. of the acidified urine contained $\frac{0{\cdot}02}{0{\cdot}82} \times 720$

$= 17{\cdot}6$ mg.

Since 17·6 mg. of ascorbic acid were contained in 720 ml. of acidified urine, this amount would also be contained in 620 ml. (720 — 100) of unacidified urine.

$$\therefore \text{ 100 ml. of unacidified urine contained } \left\{ \frac{17 \cdot 6 \times 100}{620} = 2 \cdot 8 \text{ mg. ascorbic acid.} \right.$$

PREPARATION OF SOLUTION

Forty mg. (accurately weighed) of pure dichlorophenol-indophenol dye are dissolved in 500 ml. of water. Alternatively one tablet (2 mg. of the dye ≡ 1 mg. ascorbic acid; Messrs. Hoffman—La Roche or British Drug Houses) is placed in a 25 ml. volumetric flask: about 10 ml. of warm water are added and after the tablet has dissolved the contents of the flask are cooled and diluted to the mark. The resulting solution contains 0·04 mg. of dichlorophenol-indophenol per 0·5 ml.

ANEURIN, VITAMIN B_1

PRINCIPLE

The method of estimating aneurin is that described by Wang and Harris (1939, 1943), and by Simmons and Wootton (1944). It depends on the transformation of aneurin to thiochrome and comparison by fluorescence against a similarly treated known standard of aneurin. Normal values are variously given as 33–130 I.U. (= 99–390 μg. per day), 50–80 I.U. (= 150–240 μg. per day), 30–160 I.U. (= 90–480 μg. per day). (I.U. = International Unit.)

METHOD

The specimen of urine is adjusted to approximately *p*H 3 as soon as possible. To 8 ml. of urine an equal amount of *iso*-butanol (saturated with water) is added in a 25 ml. glass-stoppered cylinder and shaken for 2 minutes. This extracted urine is used for the estimation. The butanol is discarded.

It is important that strict attention should be paid to the

following details if reliable results are to be obtained. Four cylinders are marked: A and B (blanks), T (test) and S (standard), and the following amounts of urine, etc., placed in them.

	A	B	T	Standard
Extracted urine	2 ml.	2 ml.	2 ml.	1 ml. of dilute standard.
Methanol	2 ml.	2 ml.	2 ml.	0·5 ml. water, 2 ml. of methanol.
20 % sodium hydroxide	1 ml.	1 ml.	—	—

To cylinder A is added 2 per cent. potassium ferricyanide drop by drop from a burette until the colour remains slightly more yellow than cylinder B for 30 seconds. The volume added is noted, and this tube is discarded. The remaining tubes are treated as follows:

	B	T	Standard
2 % potassium ferricyanide	—	Same amount as used in tube A.	1 drop.
20 % sodium hydroxide	—	1 ml. (mix).	1 ml. (mix).
Hydrogen peroxide 5 % (20 vol.)	5 drops.	5 drops.	5 drops.
Iso-butanol	10 ml.	10 ml.	10 ml.

All three cylinders are shaken vigorously for 2 minutes and the mixtures allowed to separate; the aqueous lower layers are removed and discarded. Four ml. distilled water are added to each cylinder and shaken for 2 minutes, allowed to separate, the water removed and 2 ml. of ethanol added to clarify. The volumes of B, T and Standard are made up to 15 ml. with redistilled *iso*-butanol, and 10 ml. of B and T are used for matching fluorescence.

Fluorimetry. Two tubes are labelled B and T. Ten ml. of the *iso*-butanol extract B are measured into tube B, and a similar quantity of T into tube T. The comparison is made in a completely dark room using an ultra-violet lamp, which is enclosed in a case fitted with a Woods glass filter. The tubes are held in front of the lamp and the fluorescence viewed down the long axis of the tubes. The bottoms of the tubes rest on a blackened platform fitted to the case of the lamp. Precautions are taken to interchange the position of the tubes and to inspect the fluorescence quickly to avoid errors due to fatigue. Standard thiochrome (S) is added to tube B drop by drop with mixing, and at the same time

an equal volume of redistilled *iso*-butanol is added to tube T, until the fluorescence in each tube is adjudged equal. Possible sources of error to be eliminated include irregularities in the size and uniformity of the tubes and the presence of fluorescing substances in the glass or the reagents. It should be noted that aspirin, quinine and other drugs may cause abnormal fluorescence. The "Hysil" test-tubes manufactured by Messrs. Chance Bros. are made of non-fluorescent glass, and are suitable for the comparison.

CALCULATION

$$\left.\begin{matrix}\mu\text{g. Aneurin in} \\ 2\text{ ml. of urine}\end{matrix}\right\} = \frac{\text{Total solution}}{\text{Amount used in test}} \times \frac{x\text{ ml. of standard used}}{\text{Volume of standard}}$$

$$= \frac{15}{10} \times \frac{x}{15} = \frac{x}{10}$$

It is usual to conduct the analysis on a sample of a 24-hour specimen of urine, and to express the result as the total output in micrograms (μg.) of aneurin for the 24-hour period.

SOLUTIONS

Preparation of Standard.—"Benerva" ampoules are used (Roche Products, Ltd). Ten mg. are dissolved in 100 ml. of N/10 hydrochloric acid in 25 per cent. alcohol. Ten ml. of this solution are diluted with N/10 hydrochloric acid and made to 1 litre. This dilute solution should be prepared fresh for use (= 1 mg. per 1000 ml. or 1 μg. per ml.).

Chapter VIII

ANALYSIS OF CALCULI

Calculi, although occasionally composed of a single constituent, are commonly mixtures of substances, and should always be cut or sawn to ascertain the presence or absence of different layers of deposits. The layers should be tested separately.

Renal Calculi. These most often contain carbonate, oxalate, calcium, uric acid or urates, ammonium salts and phosphates; more rarely cystine, "urostealith" (fatty mixtures), fibrin and xanthine.

Biliary Calculi. The following substances may be present in these stones: cholesterol, bile-pigments and calcium.

SCHEME OF TESTS

Renal Calculus

The calculus is powdered in a mortar (separate layers being treated separately). Some of the powder is treated in a test-tube with cold 2 N-nitric acid. Effervescence indicates *carbonate*. The solution is gently boiled, cooled, and filtered. Some of the filtrate is made alkaline with ammonia solution. A precipitate indicates *calcium oxalate* or *phosphate*: the phosphate precipitate being soluble in acetic acid (now added in excess), the oxalate insoluble. The presence of phosphates may be confirmed by addition of ammonium molybdate solution to the nitric acid mixture. A yellow precipitate on boiling, or an intense blue colour on the addition of aminonaphtholsulphonic acid reagent (cf., blood phosphorus), confirms phosphate. The presence of oxalate may be confirmed by its conversion to carbonate by heating some of the precipitate to red heat over a flame and treating the residue with 2 N-hydrochloric acid, when effervescence indicates *oxalate*. This solution is treated

with ammonium oxalate solution and neutralized with ammonia. A crystalline precipitate confirms *calcium*.

More of the powder is boiled in a test-tube with 2 N-sodium hydroxide ; an odour of ammonia indicates *ammonium salts*. One ml. of the filtered solution is treated with about 0·3 ml. of Folin's uric acid reagent and then with 1 ml. of sodium cyanide-urea solution. A blue colour shows *urates* or *uric acid*. The presence of these compounds may be further tested by evaporating a mixture of the powder with a little concentrated nitric acid to dryness in a porcelain dish. A red colour, becoming reddish-violet on addition of excess ammonia shows *urates* or *uric acid* : (this is the " murexide " reaction). If ammonium salts are present, the urate is probably present as ammonium urate.

Cystine burns with a pale blue flame. Its presence may be confirmed by boiling the sodium hydroxide solution of the powder with a little lead acetate : a black precipitate is given by cystine. Typical hexagonal crystals are formed if an ammoniacal cystine solution is allowed to evaporate.

Urostealith calculi are soluble in alcohol and ether. *Fibrin* burns with a " burnt feather " odour and a yellow flame, and dissolves in hot 2 N-potassium hydroxide, from which it is precipitated by acetic acid, with evolution of hydrogen sulphide.

Xanthine burns without a flame. It gives a yellow residue, turning orange with sodium hydroxide, in the murexide test.

Biliary Calculus

A little of the dry powder from a biliary calculus is well shaken with chloroform. A yellow colour, which turns red or blue on addition of the Erlich diazo reagent and excess of alcohol shows *bile-pigments*. Five ml. of the filtered chloroform solution are treated with 2 ml. of acetic anhydride and 2 drops of concentrated sulphuric acid. A green colour shows *cholesterol*.

REAGENTS REQUIRED

Concentrated nitric and sulphuric acids ; glacial acetic acid ; acetic anhydride ; alcohol ; ether ; chloroform ; 2 N-

hydrochloric acid; 2 N-ammonia; 2 N-sodium and potassium hydroxides; lead acetate (10 g. per 100 ml.); ammonium molybdate (5 g. per 100 ml.); ammonium oxalate (4 g. per 100 ml.); sodium cyanide-urea (5 g. sodium cyanide and 20 g. urea in 100 ml. of solution); Folin's uric acid reagent (see "uric acid" in blood); Erlich's diazo reagent (freshly made mixture of 10 ml. of solution "A" (1 g. sulphanilic acid + 250 ml. N-hydrochloric acid + water to 1 litre) and 0·3 ml. of solution "B" (sodium nitrite, 0·5 g. per 100 ml.)).

SOLUTIONS

2 N-*Nitric Acid.*—12·67 ml. concentrated acid diluted to 100 ml. with water.

2 N-*Ammonia Solution.*—11·4 ml. concentrated ammonia diluted to 100 ml. with water.

2 N-*Acetic Acid.*—12 ml. glacial acetic acid diluted to 100 ml. with water.

Ammonium Molybdate Solution.—5 g. ammonium molybdate dissolved in water and made to 100 ml. (see p. 55).

Aminonaphtholsulphonic Acid.—See p. 55.

2 N-*Hydrochloric Acid.*—20 ml. concentrated acid to 100 ml. with water.

Ammonium Oxalate.—Saturated (4 per cent.) solution (see p. 66).

2 N-*Sodium Hydroxide.*—8 g. per 100 ml.

Folin's Uric Acid Reagent.—See p. 10.

Sodium Cyanide—Urea Solution.—See p. 10.

Chapter IX

GASTRIC ANALYSIS

GASTRIC TEST MEAL

PRINCIPLE

The gastric mucous membrane is stimulated to secrete gastric juice by introducing alcohol (caffein solution, or gruel) into the stomach, with the purpose of imitating the normal physiological stimulus. The response may or may not be maximal. A maximal acid secretion is obtained after subcutaneous injection of histamine. The acid response is measured by estimating the concentration of " free acid " (free HCl) and " total acid " (free HCl, HCl in combination with protein buffers, butyric, lactic, and other weak organic acids) in small samples of gastric contents removed at 15-minute intervals. At the same time the presence of blood, bile, excess of mucus, lactic acid, and undigested starch may be noted.

SIGNIFICANCE OF RESULTS

Healthy subjects without gastric symptoms secrete gastric juice of widely varying acidity. Achlorhydria, even after histamine, is found in a few young persons and in an increasing proportion as age advances, up to nearly 30 per cent. at ages over 70. Achlorhydria after histamine is almost a constant finding in pernicious anaemia : achlorhydria, or low acidity, is very common in " idiopathic " microcytic anaemia. Normal or high acid values (up to 0·4 per cent. free HCl) both in resting juice and after stimulation, are very common in duodenal ulcer. There is no useful relationship between gastric acidity and the presence of gastric ulcer.

Blood in flecks may be due to trauma of the mucosa by vigorous suction ; in larger quantities it comes usually from an ulcer or a growth.

Food debris in the " resting juice," and an abnormal excess of total over free acid indicate pyloric obstruction, with decomposition of the retained gastric contents. The normal excess of " total " over " free " acid is about 10 ml. N/10 per 100 ml.

ANALYSIS OF GASTRIC CONTENTS

Gastric Acidity. Five ml. of the stomach contents are pipetted into a porcelain dish containing 2 to 3 drops of a mixture of equal volumes of Topfer * and phenolphthalein indicators.

The mixture is titrated with N/20 sodium hydroxide until a permanent orange colour is obtained (the point at which all the free acid is neutralized). Titration is then continued until the colour of the solution becomes red (total acid).

The results are multiplied by ten and thus expressed as ml. of N/10 sodium hydroxide required to neutralize 100 ml. of stomach contents.

Bile. A few ml. of the stomach contents are put into a test-tube and a few drops of N/50 iodine added. A green colour denotes bile.

Mucus. Mucus is detected by the stringy appearance of the stomach contents.

Blood. Blood is detected in the same manner as " occult blood " in faeces.

Lactic Acid. 2 ml. of stomach contents in a test-tube are shaken with 5 ml. of ether. The mixture is allowed to separate and the ether layer is removed to another tube.

The ether layer is evaporated off by immersing the tube in hot water.

The residue is dissolved in 2 ml. of water and a few drops of MacLean's reagent are added. A yellow colour indicates the presence of lactic acid.

MacLean's Reagent is a mixture of :—

100 ml. saturated mercuric chloride solution (5 per cent.)
1·5 ml. concentrated hydrochloric acid
5 g. ferric chloride.

* Topfer's indicator is a solution (0·5 g. per 100 ml.) of dimethyl yellow in alcohol.

TOTAL CHLORIDE IN GASTRIC JUICE

Volhard Method

Five ml. of gastric juice are pipetted into a 25 ml. volumetric flask and 10 ml. of N/5·85 silver nitrate in nitric acid solution (as used for the urine method) are added. The solutions are well mixed, made to the mark with water and again mixed. After 10 minutes the mixture is filtered. Ten ml. of the clear filtrate (≡ 2 ml. of gastric juice and 4 ml. of silver nitrate) are diluted with water in a porcelain dish and titrated with N/5·85 ammonium thiocyanate solution to the final permanent pink colour.

CALCULATION

1 ml. N/5·85 silver nitrate = 10 mg. NaCl.

$\therefore$ mg. NaCl in 2 ml. gastric juice = (4 − titre) × 10

$\therefore$ mg. NaCl in 100 ml. gastric juice = (4 − titre) × 10 × $\frac{100}{2}$

= (4 − titre) × 500

Chapter X

HYDROGEN ION CONCENTRATION

In pure distilled water the concentrations of hydrogen ions and of hydroxyl ions are equal. The water is "neutral." In an acid solution there is a preponderance of hydrogen ions over the concentration of hydroxyl ions. In an alkaline solution the concentration of hydroxyl ions is greater than that of hydrogen ions.

The product of the concentrations of hydrogen ions and of hydroxyl ions is the same (i.e., is a constant) in all aqueous solutions, be they acid, neutral or alkaline.

$$(H^+) \times (OH^-) = K_W$$

Pure distilled water has a concentration of one ten millionth gram of hydrogen ions per litre, i.e., $1/10^7$ or more briefly a concentration of 10^{-7} gram per litre. The concentration of hydroxyl ions in distilled water is likewise 10^{-7}. The product of the concentration of hydrogen ion and hydroxyl ion is $10^{-7} \times 10^{-7} = 10^{-14}$, the value of the constant K_W.

Hundredth normal hydrochloric acid (0·01 N-HCl) has a concentration of hydrogen ion of about 0·01 g. per litre, i.e., of 10^{-2}. The hydroxyl ion concentration will, therefore, be 10^{-12}, and the value of the product remains 10^{-14}. Likewise for hundredth normal sodium hydroxide (0·01 N-NaOH) the concentration of hydroxyl ions is 10^{-2} and of hydrogen ions 10^{-12}. And the constant K_W is in both cases 10^{-14}.

The symbol *p*H is used to denote the negative logarithm (to the base 10) of the hydrogen ion concentration, e.g., *p*H 7 for the distilled water of hydrogen ion concentration 10^{-7}, *p*H 2 for the 0·01 N-hydrochloric acid of hydrogen ion concentration 10^{-2}, and *p*H 12 for the 0·01 N-sodium hydroxide whose concentration of hydrogen ions is 10^{-12}. It forms a convenient means for designating the hydrogen ion

concentration of any solution. In most biological fluids the hydrogen ion concentrations vary only within narrow limits, and do not depart very markedly from the neutral point of *p*H 7.

The colorimetric determination of *p*H is dependent on the use of indicators, organic compounds which change colour with change in hydrogen ion concentration. The changes in colour of indicators take place over definite and fairly narrow ranges of hydrogen ion concentration, within which intermediate shades of colour corresponding to different values of *p*H can be recognized. The following is a list of the indicators most commonly used in biological work :

Indicator	p*H range and colour change*
Thymol blue	red 1·2 to 2·8 yellow.
Topfer's indicator (dimethyl yellow)	red 2·8 to 4·5 yellow.
Methyl orange	red 2·9 to 4·6 yellow.
Bromphenol blue . . .	yellow 3·0 to 4·6 blue.
Methyl red	red 4·2 to 6·3 yellow.
Brom thymol blue . . .	yellow 6·0 to 7·6 blue.
Phenol red	yellow 6·8 to 8·4 red.
Thymol blue	yellow 8·0 to 9·6 blue.
Phenolphthalein . . .	colourless 8·2 to 10·0 red.

METHOD

The *p*H of an unknown liquid may be determined by adding 1 ml. of a dilute solution of the indicator to 10 ml. of the liquid in a test-tube. The tube is stoppered, the contents mixed, and the colour compared with those in a standard set of buffered solutions of known *p*H containing the same amount of indicator. In the case of coloured solutions (e.g., urine) it is convenient to compensate for the colour by placing a tube containing the properly diluted coloured solution behind the standard indicator tube, and a tube of water behind that containing coloured solution plus indicator. A special comparator apparatus is best used for this purpose. An approximate *p*H value for the liquid can be obtained by noting the colour produced with one or more indicators and comparing with the table. Thus, if urine is yellow to brom

thymol blue and is yellow or orange yellow to methyl red, its *p*H is probably in the neighbourhood of 6·0. For fuller discussion and instructions see: Britton (1942), & Clark (1928). A convenient comparator, complete with tubes of standard buffers with indicators, for the colorimetric determination of *p*H is obtainable from Messrs. British Drug Houses and Messrs. Hopkins & Williams. The B.D.H. Capillator set is a useful instrument for *p*H measurements of very small, as well as large, quantities of liquid.

SOLUTIONS

The indicators mentioned above may conveniently be purchased in ready-made solutions. If it is desired to prepare them from the solid dyes, then 0·1 g. of thymol blue, bromphenol blue, bromthymol blue or phenol red should be dissolved in 20 ml. of warm alcohol and diluted to 100 ml. with water. Preparation of Topfer's indicator is given on p. 111, and of methyl orange, methyl red and phenolphthalein on p. 141.

Chapter XI

SPECTROSCOPIC PROCEDURES

In many cases it is useful to examine solutions spectroscopically to identify the pigments present. Many pigments give characteristic dark absorption lines in the visible part of the spectrum, and these lines may be detected and their position in the spectrum approximately determined by direct visual examination with some sort of simple spectroscope.

DIRECT-VISION SPECTROSCOPE

A form of this instrument is illustrated.

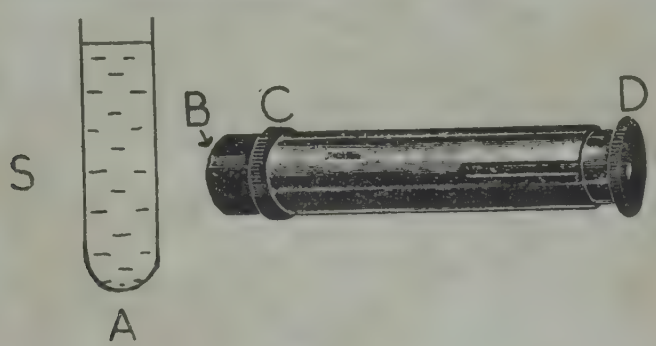


Fig. 5.

Light from a source S travels through the solution A and reaches the eye after spectroscopic resolution by means of a prism and lens system in the instrument. The size of the slit B may be adjusted by means of the screw C and the spectrum is focused by moving the eyepiece, D. For the examination of solutions, the eyepiece is placed close to the eye, and the apparatus pointed at a bright light, preferably daylight. The spectrum is sharply focused, and the test-tube or glass cell containing the solution is placed so that it touches the slit B. The spectrum should now be clearly and sharply visible; if not, the slit B, and the eyepiece D, are adjusted until this condition is attained. The dark bands due to the pigment ought now to be visible.

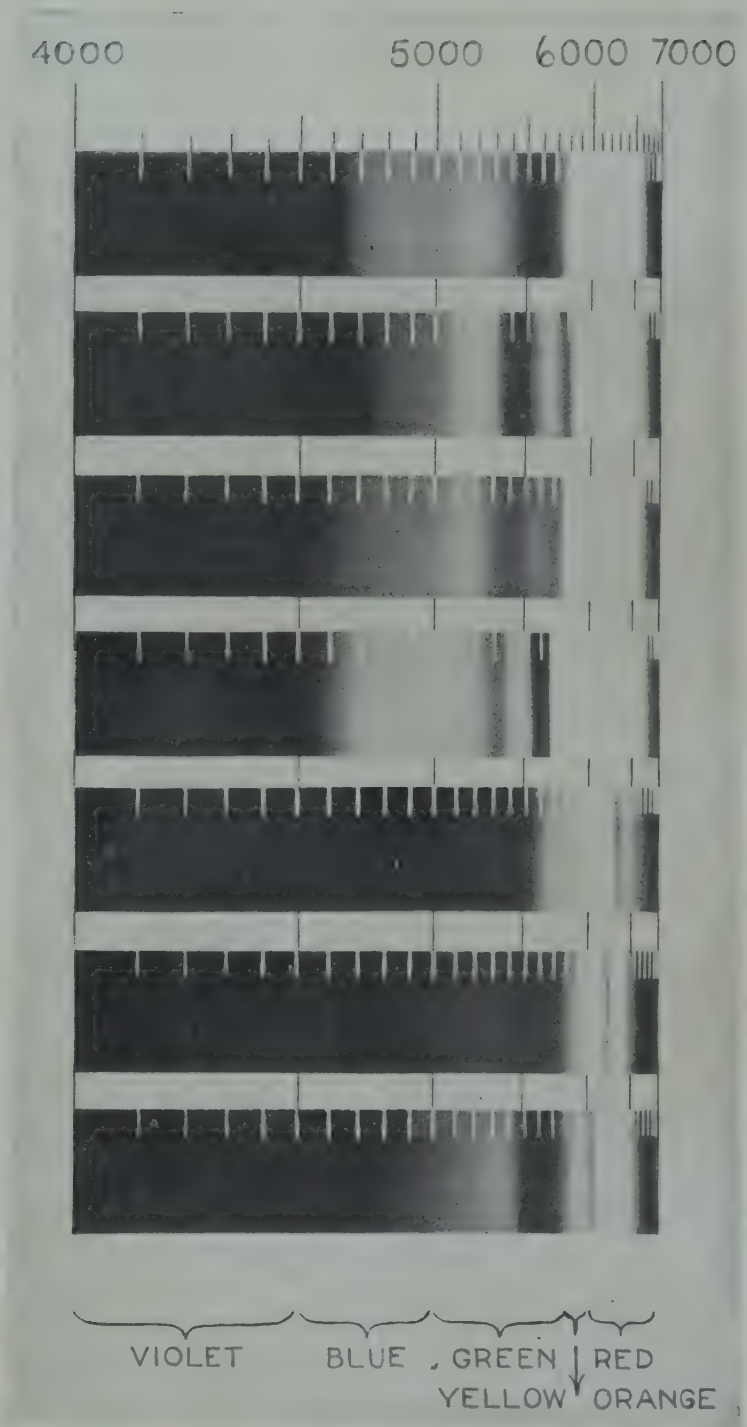
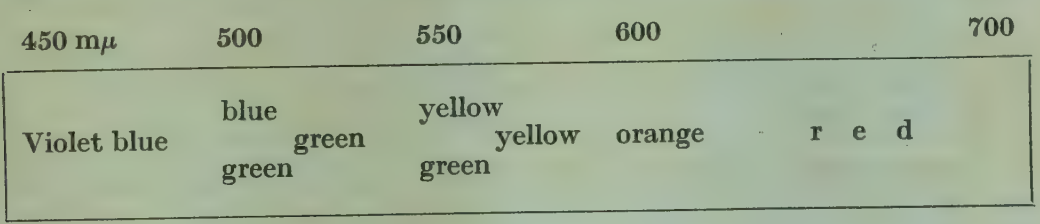


FIG. 6.—Absorption spectra of haemoglobin and its derivatives.
(From Thorpe's *Biochemistry*.)

[*To face p.* 117

The following are characteristic :—

Pigment	No. of bands	Position on the spectrum. (Wave-length in mμ.)
Reduced haemoglobin	1	565
Oxyhaemoglobin	2	540, 578
Carboxyhaemoglobin	2	535, 572
Methaemoglobin	4	500, 540, 579, 630
Alkaline methaemoglobin	2	541, 580
Sulphaemoglobin	3	540, 578, 618
Haemochromagen	2	526, 557
Acid haematin	5	505, 540, 580, 638, 650 (in dilute HCl)
Alkaline hæmatin	1	607
Acid porphyrin	2	554, 600
Alkaline porphyrin	4	504, 539, 576, 622
Stercobilin (urobilin)	1	490



METHODS

Blood

Blood is diluted 1 in 5, or 1 in 10, with water, and cell membranes centrifuged out or allowed to settle. The clear solution is examined in a glass cell or tube. It is important that the greatest possible depth or concentration of solution (consistent with visibility) should be examined, and that a careful search should be made (with varying depths or concentrations of solution) for any bands in the red part of the spectrum (620–630 mμ). The bands due to met- or sulphaemoglobin, present sometimes in blood from patients treated with drugs (e.g., sulphanilamide and its derivatives), are not always easy to detect. If such bands (at approx. 620–630 mμ) are seen, the solution should be treated with a drop of yellow ammonium sulphide. A band due to methaemoglobin will then disappear; if sulphaemoglobin is present its band persists. For comparisons, laked blood may

be treated with a little potassium ferricyanide solution, which will cause methaemoglobin to be formed. A sample of sulphhaemoglobin may be prepared from blood (10 ml. of 1/100 dilution), phenylhydrazine hydrochloride solution (0·1 ml. of a 0·1 per cent. solution), and a drop of water saturated with hydrogen sulphide.

Urine may be directly examined (after filtration or centrifuging) for urobilin, porphyrin, and haemoglobin pigments.

Faeces

(*a*) **For Stercobilin.** Approximately 1 g. of faeces in a test-tube is shaken well with 15 ml. of acid alcohol (1 ml. of conc. hydrochloric acid per 100 ml. of alcoholic solution). After some hours, the supernatant liquid (with dilution, if necessary, with acid alcohol) is examined spectroscopically. The acid alcohol converts stercobilinogen into stercobilin.

(*b*) **For Blood.** About 1 g. of faeces is well shaken in a glass-stoppered cylinder with 10 ml. of water. Ten ml. of glacial acetic acid and 20 ml. ether are now added, and the mixture carefully shaken. The emulsion is allowed to separate (with addition, if necessary, of a little more ether or water). The ether layer is examined for alkaline porphyrin and is then decanted and shaken with 10 ml. of 2 N-hydrochloric acid. Each layer is examined. The ether may contain acid haematin (from *blood*) and chlorophyll, and the aqueous part, acid porphyrin. Stercobilin may be present in both layers.

THE QUANTITATIVE ESTIMATION OF CARBON MONOXIDE IN BLOOD
HARTRIDGE REVERSION SPECTROSCOPE

In cases of carbon monoxide poisoning, the blood may appear a bright carmine in colour, due to the presence of carboxyhaemoglobin. The spectrum of carboxyhaemoglobin is similar to that of oxyhaemoglobin, but the bands (α and β) in the green are shifted slightly towards the violet end of the spectrum. This shift, though difficult to detect with a simple

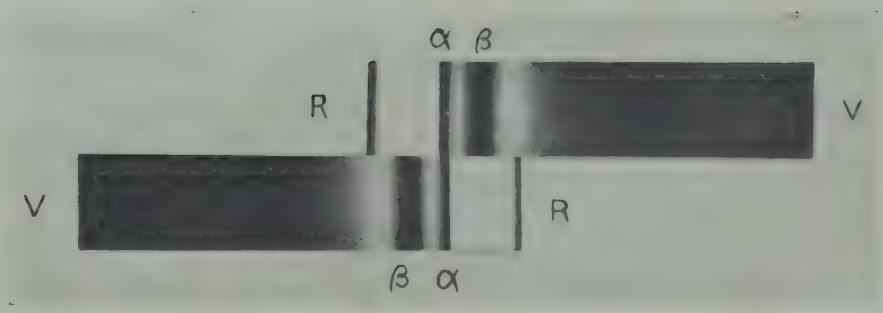


FIG. 7.—Spectrum of oxyhaemoglobin in Hartridge reversion spectroscope; adjusted to make α lines correspond.

(From Thorpe's *Biochemistry*.)

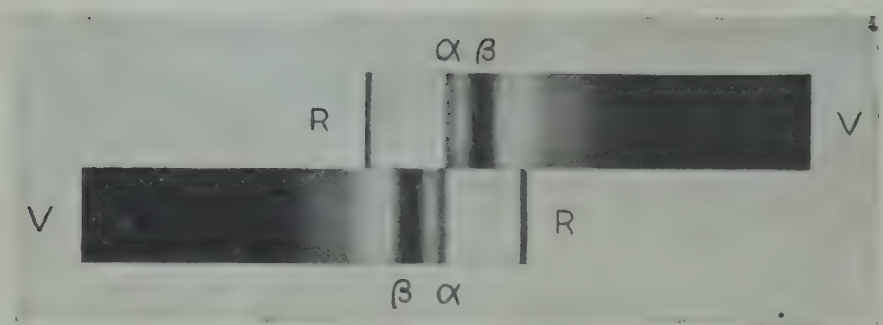


FIG. 8.—Spectrum of carboxyhaemoglobin in Hartridge reversion spectroscope; showing shift of α bands from their position in oxyhaemoglobin.

(From Thorpe's *Biochemistry*.)

[*To face p.* 119

spectroscope, may be quantitatively measured by means of the Hartridge Reversion Spectroscope. In this instrument light from a source passes through a solution in such a way that two spectra are formed, one immediately above the other.

The spectra are also reversed with respect to one another, and the position of one of them can be altered longitudinally by moving a micrometer screw. If a glass cell filled with a solution of oxyhaemoglobin is placed in the path of the light the corresponding lines (α) in the spectra may be made to coincide (fig. 7). If, now, the cell is replaced by one containing some carboxyhaemoglobin, the bands will appear shifted with respect to one another (fig. 8). By adjusting the micrometer screw until coincidence is again obtained, the extent of the shift is measured and can be related to the percentage of carboxyhaemoglobin by reference to a graph, obtained as follows :—

Blood is diluted with ammonia solution (4 ml. of conc. ammonia per litre in water). The dilution of blood (usually 1 in 20) must be such that, with the cell used, the distance between the α and β haemoglobin bands is approximately equal to the width of one of them. The solution so formed is saturated with oxygen to give 100 per cent. oxyhaemoglobin and with coal gas to give 100 per cent. carboxyhaemoglobin solutions. The two cells A and B are then placed face to face so that the light traverses them both. Cell A is first filled with 100 per cent. oxyhaemoglobin and B with ammonia solution. A reading is taken at coincidence of the α haemoglobin bands. Cell A is now emptied and refilled with 100 per cent. carboxyhaemoglobin solution and another reading taken, at coincidence. For 50 per cent. saturation of carboxyhaemoglobin, cell A is filled with a mixture of equal volumes of dilute ammonia and 100 per cent. carboxyhaemoglobin solution and cell B with a similar mixture of ammonia and 100 per cent. oxyhaemoglobin solution. For

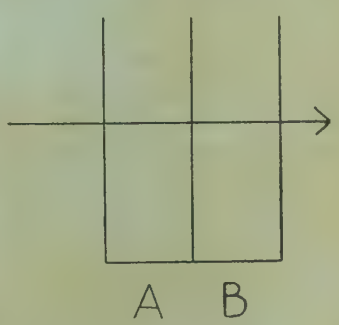


FIG. 9.

25 per cent. saturation of carboxyhaemoglobin, cell A contains a mixture of 25 volumes of 100 per cent. carboxyhaemoglobin and 75 volumes of dilute ammonia, while cell B is filled with a mixture of 75 volumes of oxyhaemoglobin and 25 volumes of dilute ammonia. Similarly, points for other concentrations of carboxyhaemoglobin are obtained. In each case, coincidence of the α line is obtained, and the micrometer reading noted. The readings for the various concentrations are subtracted from that for 100 per cent. oxyhaemoglobin, and the results plotted as shown.

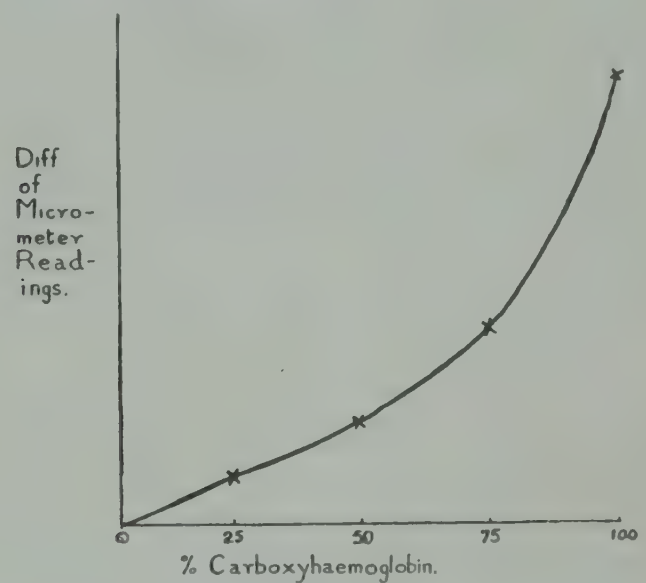


FIG. 10.

For the estimation, a 100 per cent. oxyhaemoglobin solution of the above dilution is placed in a cell, and coincidence obtained. The cell is now replaced by another containing the suspected blood, similarly diluted, and the amount of shift (x) necessary to obtain coincidence is measured. It is advisable to check the instruments, also, with a 100 per cent. carboxyhaemoglobin solution. The results obtained for this and for 100 per cent. oxyhaemoglobin should agree with the standard graph. When this has been done, the amount of shift (x) given by the suspected blood can be related to percentage saturation with carboxyhaemoglobin by simple reference to the curve.

Chapter XII

TESTS OF FUNCTION

THE GLUCOSE TOLERANCE TEST

The patient is fasted for 12 hours or longer. The " fasting blood sugar " is estimated by the method described. Immediately after the blood has been taken, a solution of 50 g. of glucose in 250 ml. of water is given (for youthful subjects,

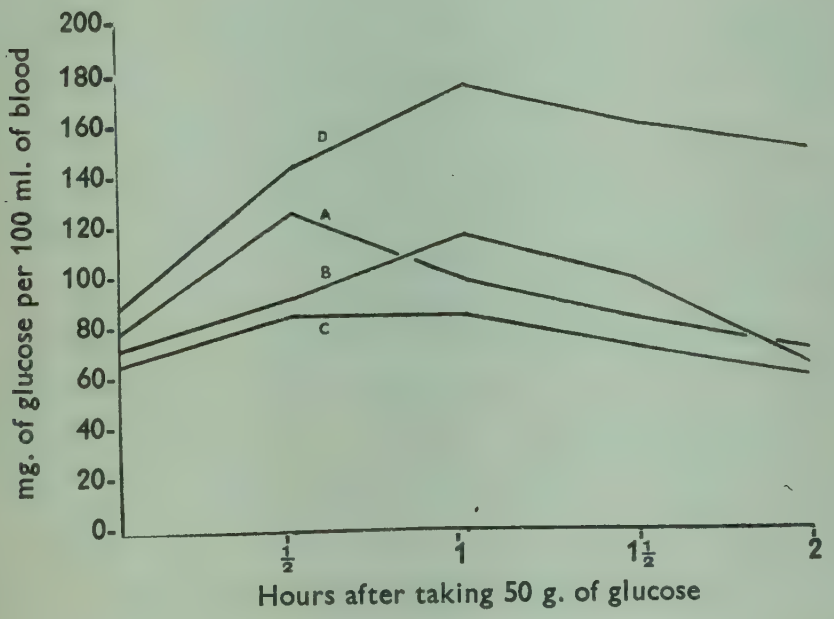


Fig. 11.—Glucose Tolerance Tests.

A & B. Normal.
C. Increased tolerance (e.g., Addison's disease).
D. Decreased tolerance (Diabetes).

the amount of glucose should be 1 g. per 3 lb. of body weight). Blood sugar estimations are then made at ½, 1, 1½, and 2 hourly intervals after the administration of the glucose. Samples of urine are taken before the test and 2–4 times during its course. In normal persons, the blood-sugar will not generally rise above 160 mg. per 100 ml., and the urine will not give a positive test for sugar. The blood glucose level

will show a sharp peak after ½ to 1 hour, falling to or nearly to normal at the end of 2 hours. In diabetic cases, the blood glucose may rise to very high levels (300–600 mg. per 100 ml.) and *only returns very gradually to normal.* Sugar is almost always present in the urine.

GALACTOSE TOLERANCE TEST

Transformation of Non-glucose Sugars into Glycogen

Galactose, in common with various other sugars, e.g., laevulose, xylose, is not metabolized directly in the body as is the case with glucose, but must first be transformed into glycogen. The capacity of the liver for transforming these other sugars into glycogen has been found to be altered in certain conditions of impaired liver function, and the rate of removal of the sugar in question from the blood forms the basis of an estimate of the capacity of the liver for transforming the non-glucose sugar.

By the galactose tolerance test it is possible only to detect fairly severe degrees of liver damage. The test, in conjunction with other investigations (e.g., Bilirubin and Phosphatase determinations) may be useful in differentiating between obstructive and non-obstructive jaundice.

MODE OF ADMINISTRATION

As most commonly employed, the test consists in the oral administration of 40 g. of galactose, followed by analysis of the urine passed in the succeeding five hours to determine the total amount of galactose excreted. If the liver (and to a lesser extent, other tissues) has metabolized the administered galactose to such an extent that less than 3 g. is excreted in the five hours following ingestion, its functional capacity is held to be unimpaired. The excretion of more than 3 g. of galactose is considered to indicate subnormal hepatic function.

A more exact procedure is to inject the galactose solution and estimate the rate of its disappearance from the blood.

EXPERIMENTAL PROCEDURE

A solution of galactose (50 g. per 100 ml. of solution) is prepared and sterilized by filtration and steaming.

Graded Doses of Galactose. The patient receives no breakfast. An amount of solution equivalent to 0·5 g. of galactose per kg. of body-weight—e.g., 60 ml. of the 50 per cent. solution for a 60 kg. person—is injected intravenously from a 100 ml. syringe, the injection being given slowly over about five minutes.

Standard Dose of Galactose. The injection of 50 ml. of a 50 per cent. solution of galactose has given, with adults, results indistinguishable from those obtained with graded doses. This is a much more convenient amount to inject, because most hospital wards have 50 ml. syringes, whereas 100 ml. syringes are usually special equipment.

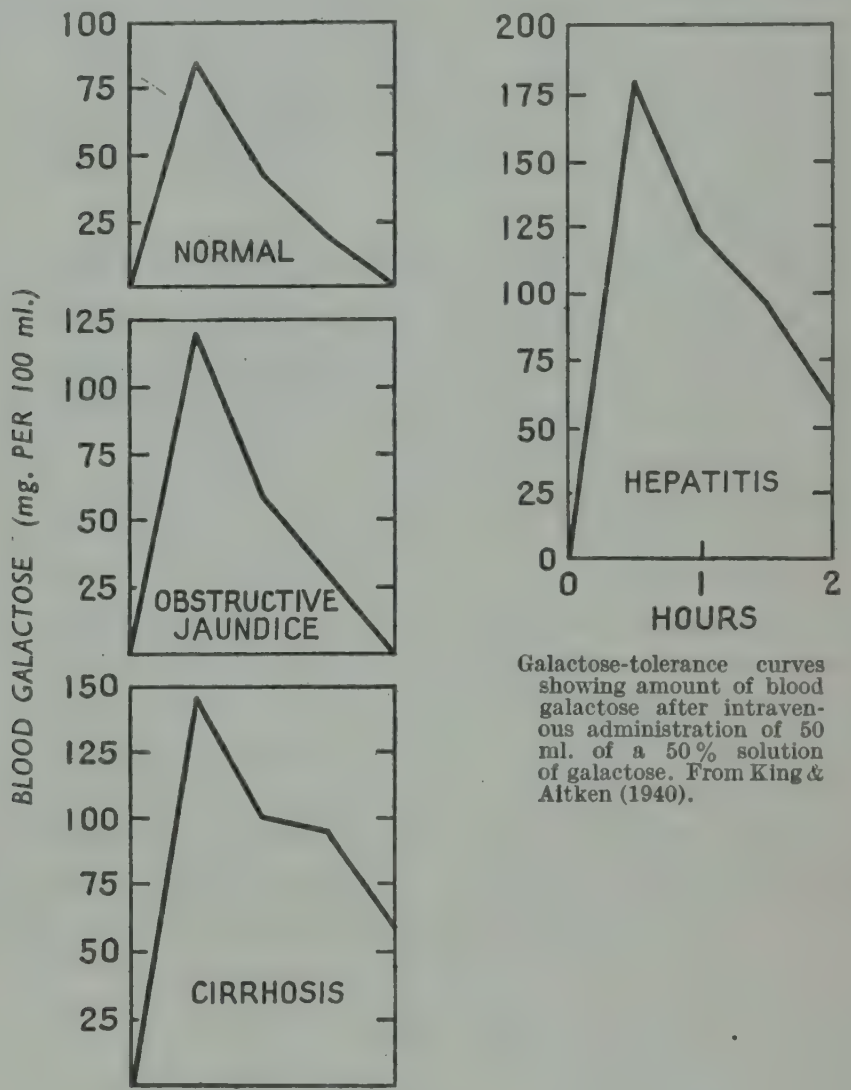
Samples of Blood. The first sample of blood is taken immediately after the administration of the galactose (5-minute sample), the second sample at ½ hour, and further samples at 1, 1½, and 2 hours. A sample taken before the galactose administration may be substituted for that at 5 minutes. This resting sample is a useful check on the analytic method because it should contain no galactose.

Analysis. The samples of blood (0·2 ml. of capillary blood) are washed into centrifuge tubes containing 2·2 ml. of isotonic sodium sulphate and 0·3 ml. of 10 per cent. sodium tungstate. The estimation is completed as described on p. 23.

RESULTS

The galactose values are plotted against time and for a normal person usually give a curve which begins about 200 mg. of galactose per 100 ml. of blood, falls steeply during the first hour, and reaches a figure between 0 and 10 mg. per 100 ml. at the end of two hours. In most cases of obstructive jaundice the curve of blood galactose follows the same course, there being little or no galactose left in the blood at the end of two hours. In conditions of liver damage the level of galactose in the blood does not

return to the normal level within the two hours. The level of blood galactose is extremely variable in the first (5-minute) specimen. This is inevitable because of the variable time which it takes to inject the galactose in



Galactose-tolerance curves showing amount of blood galactose after intravenous administration of 50 ml. of a 50% solution of galactose. From King & Aitken (1940).

FIG. 12.

different people. The $\frac{1}{2}$, 1, and $1\frac{1}{2}$ hour specimens show greatly different values for different people, but on the whole the curve of blood galactose appears to be lower in the normal, non-hepatic, and obstructive jaundice cases than it is in the toxic and infective groups. The results should not be considered to have any final diagnostic significance.

however, and should only be interpreted against the background of the clinical and other biochemical findings.

INTRAVENOUS HIPPURIC ACID TEST

PRINCIPLE

The detoxicating power of the liver is assessed by administering benzoic acid (as the sodium salt) which is conjugated with glycine, in the liver, to form hippuric acid, and excreted as such by the kidneys. The specificity of the test for hepatic as opposed to renal function is not yet certain, and a simultaneous urea clearance test should be run in parallel.

METHOD

Administration.—Twenty ml. of an 8·85 per cent. solution of sodium benzoate, = 1·77 g. sodium benzoate, equivalent to 1·5 g. benzoic acid, is injected slowly into an arm vein. Immediately after the injection, and exactly one hour later, the bladder is emptied. The first specimen is discarded, and the hourly specimen used for the analysis. In order to ensure an adequate urine output, the patient is given a pint of water to drink after the injection.

Estimation (Weichselbaum and Probstein, 1939).—The urine volume is measured, and if it exceeds 120 ml. is acidified with a little glacial acetic acid, evaporated down and again measured. If the urine is very heavily pigmented, a little charcoal is added. The warm urine is then saturated with salt, 30 g. per 100 ml., heated and filtered. The filtrate is acidified with 50 per cent. sulphuric acid, till acid to Congo Red ; and is left to stand in the cold, preferably overnight, until crystallization is complete. Crystallization can sometimes be induced by scratching the sides of the vessel with a glass rod, or by adding a small crystal of hippuric acid. The crystals are then filtered off by suction, washed with cold 30 per cent. sodium chloride and dissolved in distilled water by warming. The solution is titrated against 0·5 N-sodium hydroxide, using phenolphthalein as indicator.

CALCULATION

Allowance must be made for the solubility of hippuric acid in urine saturated with salt at room temperature (0·123 g. per 100 ml. ≡ 0·1 g. sodium benzoate).

1·0 ml. 0·5 N-NaOH ≡ 0·072 g. C_6H_5COONa

∴ Weight of hippuric acid excreted *

$$= \text{ml. NaOH used} \times 0{\cdot}072 + \left(0{\cdot}1 \times \frac{\text{Vol. urine}}{100}\right).$$

* Expressed as g. sodium benzoate.

Normal range (Quick, Ottenstein and Weltchek, 1938)—

0·86–1·12 g. expressed as sodium benzoate.
0·7 –0·95 g. „ „ benzoic acid.

UREA CLEARANCE TEST

The " urea clearance test " (devised by Moeller, McIntosh and Van Slyke) aims to obtain a measure of the efficiency with which the kidney excretes urea. It shows the response of the kidney to the stimulation of the actual amount of urea in the blood. The clearance is defined in terms of the number of millilitres of blood " cleared " of urea per minute.

It has been found that with large volumes of urine (above 2 ml. per minute) the output of urea depends upon, and is directly proportional to the level of urea in the blood. Where the rate of urine excretion falls below 2 ml. per minute this direct relationship no longer holds and the output of urea is now found to vary, not only with the blood urea but also with the square root of the volume of urine.

In order to obtain a maximum physiological stimulation of the kidney, urea is given to the patient with a liberal drink of water before commencing the test. In cases where the blood urea is already high the water alone is given.

The necessary data are the urea concentration of the blood and of the urine, and the volume of urine excreted in the given time.

The normal " maximum clearance " (when the flow of

urine is above 2 ml. per minute) is usually about 40 per cent. greater than the " standard " clearance (flow of urine less than 2 ml. per minute). The average normal values are as follows :—

> For the " maximum clearance " an average of 75 ml. of blood per minute with variations from 64 to 99 ml., and for the " standard clearance " an average of 54 ml. with variation from 40 to 68 ml.

Nephritic patients with diminishing renal efficiency show a decreased " clearance " (down to about 40 per cent.) before the blood urea and the blood creatinine begin to rise ; on the other hand, the maximum specific gravity of the urine is often diminished before the urea clearance test gives abnormal results. When the clearance falls to 5 per cent. the symptoms of uraemia usually appear.

METHOD

The patient is given no breakfast and nothing to drink in the early morning other than water if desired.

The test is carried out in the forenoon as follows :—

0 hours :	Empty bladder Give 15 grams urea in 500 ml. water, if the blood urea is not raised. (If blood urea is known to be raised, or likely to be raised, give 500 ml. water only.) *		No.
½ hour :	Take blood for urea estimation . .	Blood	1
1 hour :	Empty bladder † — send complete specimen to laboratory . . .	Urine	1
1½ hours :	Take blood for urea estimation . .	Blood	2
2 hours :	Empty bladder † — send complete specimen to laboratory . . .	Urine	2

* The patient's blood urea should usually be determined a day or two before the Clearance Test is carried out.

† The specimens of urine must be taken exactly at the times specified. If for any reason it is found necessary to deviate from the 60-minute periods, the exact time (in minutes) during which the urine has collected (i.e., since the previous evacuation) should be specified.

If the excretion of urine is at a rate greater than 2 ml. per minute, the so-called " maximum urea clearance " is calculated according to the following formula :

$$C_m = \frac{U\,V}{B}$$

where C_m = maximum urea clearance, i.e., volume of blood " cleared " of urea in 1 minute during moderate diuresis.

U = concentration of urea in urine.

V = volume of urine per minute.

B = blood urea (mg. per 100 ml.).

If the excretion of urine is at a rate less than 2 ml. per minute, the so-called " standard urea clearance " is calculated from the following formula :

$$C_s = \frac{U}{B}\sqrt{V}$$

where C_s = standard urea clearance,

and U, V, and B are the same as above.

The result of the test is usually expressed as a percentage of the average normal clearance, which is taken to be 75 ml. of the blood per minute if the rate of urine excretion is above 2 ml. per minute (C_m), and 54 ml. blood if the rate is below 2 ml. per minute (C_s).

C_m is therefore multiplied by $\frac{100}{75}$ or 1·33,

and C_s by $\frac{100}{54}$ or 1·85.

Normal kidneys show a fairly wide variation in clearance, but figures below 70 per cent. usually indicate impaired renal efficiency. Examples are given below.

Urea Clearance Test on Normal Individual

	1st hour	2nd hour	3rd hour
Blood Urea	67·4 mg.	71·9 mg.	71·4 mg.
Urine Urea	1950 mg.	3000 mg.	3930 mg.
Urine Volume	120 ml.	52 ml.	35 ml.
CLEARANCE	$C_s = \frac{1950}{67 \cdot 4}\sqrt{\frac{120}{60}}$	$C_s = \frac{3000}{71 \cdot 9}\sqrt{\frac{52}{60}}$	$C_s = \frac{3930}{71 \cdot 4}\sqrt{\frac{35}{60}}$
Blood cleared of Urea per minute	40·9 ml.	38·85 ml.	41·9 ml.
% of Normal Function	$(C_s \times 1 \cdot 85) = 75 \cdot 8$	$(C_s \times 1 \cdot 85) = 71 \cdot 7$	$(C_s \times 1 \cdot 85) = 77 \cdot 4$

Urea Clearance Tests on Clinical Cases

1. (*m. 19*) *Nephritis*, blood urea 34 mg. per 100 ml. (15 g. urea in 500 ml. water administered):

	1st hour	2nd hour
Blood Urea	63·0 mg. per 100 ml.	69·0 mg. per 100 ml.
Urine Urea	1275 „ „	1770 „ „
Urine Volume	96 ml.	70 ml.
CLEARANCE	$C_s = \frac{1275}{63}\sqrt{\frac{96}{60}}$	$C_s = \frac{1770}{69}\sqrt{\frac{70}{60}}$
Blood cleared of Urea per minute	= 25·6 ml.	= 27·7 ml.
% of Normal Function	$(C_s \times 1 \cdot 85) = 47$	$(C_s \times 1 \cdot 85) = 51$

Average = 49 % of Normal Function.

2. (*f. 18*) *SubacuteNephritis*, blood urea 33·8 mg. per 100 ml. (15 g. urea in 500 ml. water administered):

	1st hour	2nd hour
Blood Urea	87·3 mg. per 100 ml.	102 mg. per 100 ml.
Urine Urea	900 „ „	895 „ „
Urine Volume	116 ml.	134 ml.
CLEARANCE	$C_s = \frac{900}{87 \cdot 3}\sqrt{\frac{116}{60}}$	$C_m = \frac{895}{102} \times \frac{134}{60}$
Blood cleared of Urea per minute	= 14·3 ml.	= 19·6 ml.
% of Normal Function	$(C_s \times 1 \cdot 85) = 27$	$(C_m \times 1 \cdot 33) = 26$

Average = 26·5 % of Normal Function.

3. (*f. 67*) *Uraemia*, blood urea 193 mg. per 100 ml. (500 ml. water administered):

Blood Urea . . .	209 mg. per 100 ml.	209 mg. per 100 ml.
Urine Urea . . .	615 „ „	615 „ „
Urine Volume . . .	100 ml.	95 ml.
CLEARANCE . . .	$C_s = \frac{615}{209}\sqrt{\frac{100}{60}}$	$C_s = \frac{615}{209}\sqrt{\frac{95}{60}}$
Blood cleared of Urea per minute . . .	$= 3 \cdot 8$ ml.	$= 3 \cdot 7$ ml.
% of Normal Function .	$(C_s \times 1 \cdot 85) = 7 \cdot 0$	$(C_s \times 1 \cdot 85) = 6 \cdot 9$

Average $= 6 \cdot 95$ % of Normal Function.

4. (*f. 54*) *Pyelonephritis*, *Uraemia*, blood urea 250, rising to 640 mg. per 100 ml., urine albumin 600 mg. per 100 ml.

Post Mortem: abscesses in both kidneys, subacute diffuse nephritis. (500 ml. water administered):

Blood Urea . . .	378 mg. per 100 ml.	250 mg. per 100 ml.
Urine Urea . . .	902 „ „	892 „ „
Urine Volume . . .	16 ml.	10 ml.
CLEARANCE . . .	$C_s = \frac{902}{378}\sqrt{\frac{16}{60}}$	$C_s = \frac{892}{250}\sqrt{\frac{10}{60}}$
Blood cleared of Urea per minute . . .	$= 1 \cdot 2$ ml.	$= 1 \cdot 5$ ml.
% of Normal Function .	$(C_s \times 1 \cdot 85) = 2 \cdot 3$	$(C_s \times 1 \cdot 85) = 2 \cdot 7$

Average $= 2 \cdot 5$ % of Normal Function.

GASTRIC TEST MEALS

Test meals are tests of gastric function. Through them a study is made of the gastric secretion—of the quality and quantity of the gastric juice in relation to its different constituents. The presence of abnormal constituents is noted, and the time it takes the meal to leave the stomach. The factors influencing gastric secretion are psychic, humoral and chemical. Tests of function are based on the response to chemical and humoral stimuli. The chemical stimulus used is usually a simple meal of dry toast or oatmeal gruel, or of dilute alcohol. This type of stimulus may be called "the physiological stimulus." Humoral stimulus is obtained

usually by injection of a small amount of histamine. This may be called " the pharmacological stimulus."

Histamine Test

Histamine has definite advantages over the ordinary test meal. It evokes a maximum response, and often produces acid secretion where the ordinary test meals fail completely. It is independent of the psychic factors involved in the taking of any ordinary meal. It adds nothing to the stomach so that a pure juice, undiluted and uncontaminated, is obtained; and no neutralization of the acid can take place through food constituents.

The Combined Alcohol and Histamine Tests

Alcohol as a physiological stimulant has several advantages over gruel, charcoal biscuit or dry toast. It is much less objectionable to take, is easily swallowed, and evokes a feeling of pleasure instead of distaste on the part of the subject.

The gastric juice obtained is ideal for analysis. It is thin and easily pipetted. No suspended food material obscures the colour and renders it turbid. The presence of bile, mucus, and altered blood are much easier to detect, and the end-points in the titration are sharper and better defined.

Alcohol seems to cause a more ready response of gastric secretion than the other test meals. The curve of acidity rises more sharply. There is not the preliminary drop following the resting specimen which is observed in most fractional test meals, and is probably due to the neutralization of the first juice secreted, by the food stuff administered. Alcohol does not neutralize any of the acid. It is a neutral substance and only dilutes the juice. Further, a sufficient amount can be given in much less volume so that the dilution factor is small. Instead of a pint of gruel, only 50 ml. of aqueous alcohol are usually given.

The response to alcohol is much more quickly over—partly because it does not remain so long in the stomach. The

purely liquid meal is soon evacuated. Most alcohol test meals show a decline in acidity by the end of an hour. A test meal of suspended solids takes 1½ to 2 hours.

Administration of histamine following the alcohol effect gives a test which combines the advantages of the simple physiological type of stimulus with those very definite ones possessed by the pharmacological type of stimulus. And it allows of comparing in the same subject the two types of response.

The combined test (which is really two tests in one) is quicker than an ordinary test meal and yields more valuable information.

METHOD OF THE COMBINED TEST

The patient is given a light supper the night previous. The tube is passed and the stomach emptied as far as possible of its resting juice. Fifty ml. of 7 per cent. alcohol are then given the patient to drink. About 10 ml. of fluid is withdrawn at the end of 15, 30, 45 minutes and 1 hour. 0·5 mg. of histamine is then given by intramuscular injection. Samples of juice are taken at ½ and 1 hour. The total time consumed by the test is thus 2 hours.

Resting juice should be studied in relation to its volume and nature. Attention should be paid to the presence of a foul odour and to the presence of abnormal constituents such as altered blood and lactic acid.

The *test meal samples* of juice should be studied particularly in regard to their free and total acidity. The passage of fluid from stomach to duodenum—and hence the condition of the pylorus, is deduced from the shape of the curve. Regurgitation from the duodenum is looked for in the presence of bile.

INTERPRETATION

1. *Gastric Carcinoma.* There is usually an absence of free HCl during the alcohol portion of the test. Histamine may, or may not, confirm this achlorhydria. The combined

acid usually turns out to be organic acid; a strong test for lactic acid is found. Blood is frequently present, and mucus is excessive. The foul smell of the resting juice is characteristic.

2. *Gastric Ulcer.* The results are very irregular, but are usually fairly like the normal. The curves for free and total HCl are in many cases within the normal range, although high results are often encountered. Mucus and bile may or may not be present. The presence of altered blood—" coffee-grounds "—is significant.

Stenosis is evidenced by an increasing acidity which does not show the normal tendency to fall, giving a " plateau " type of curve. The presence of blood with an increasing acidity curve indicates a gastric rather than a duodenal ulcer.

No differentiation can be made on the basis of the curve between a simple hyperchlorhydria and one due to an organic lesion. Only the presence of blood will indicate the latter.

3. *Duodenal Ulcer.* Hyperchlorhydria is usually present; it may be very marked. If the ulcer be just below the pylorus, stenosis may result. The acid curve will then be very high and will not fall for some time. Other findings are not of note.

4. *Achlorhydria.* True achlorhydria is found in pernicious anaemia. No secretion is brought out by either the alcohol or the histamine.

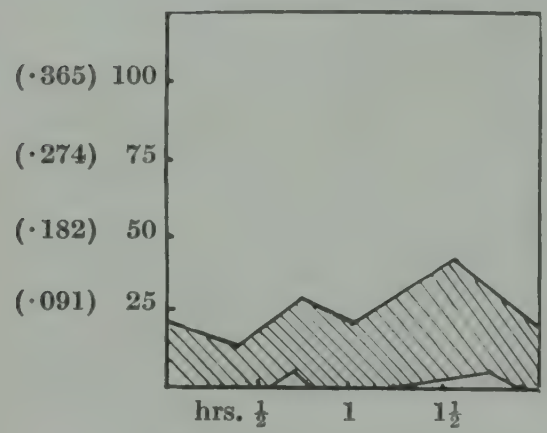
Apparent achlorhydria, on the other hand, shows an absence of free HCl in the alcohol part of the test, but the histamine successfully stimulates its secretion.

5. *Gastro-enterostomy,* if successful, relieves hyperchlorhydria completely. The free HCl is very low or absent, due to the constant regurgitation of bile. The curve of total acidity is a low normal.

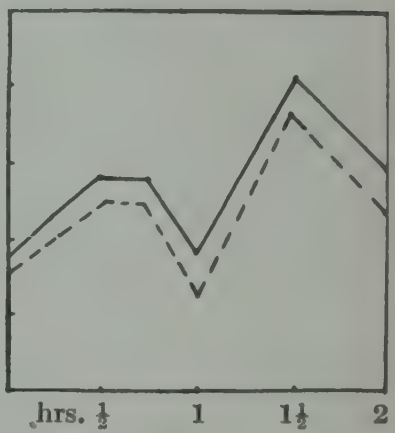
6. *Partial Gastrectomy.* The curve of free acidity is low. Bile is frequently present. The stomach empties quickly. If the operation has been unsuccessful the acidity curves will continue to be high. A routine test meal should be conducted within a few weeks of the operation.

FRACTIONAL TEST MEALS

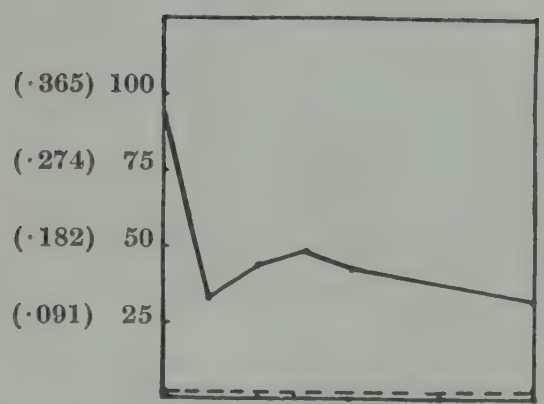
Alcohol and Histamine : Types of Response



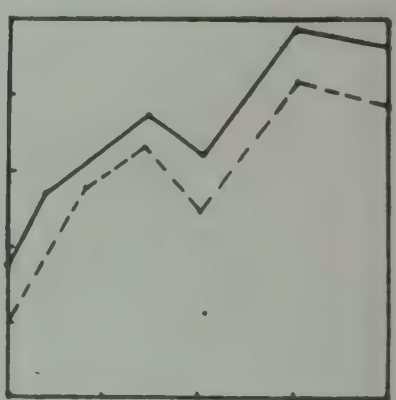
Normal : free HCl, area within which most values fall with normal persons.



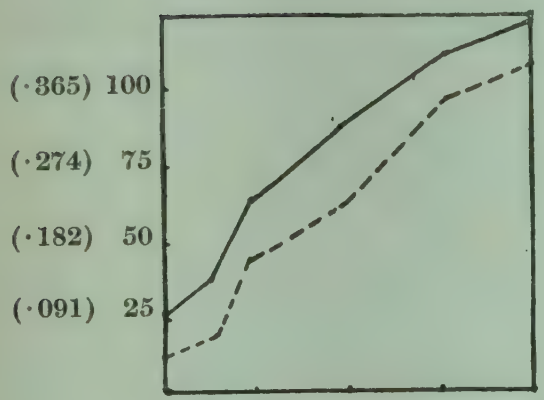
Gastric ulcer : fairly high free and total HCl ; small amount of mucus ; no bile or blood.



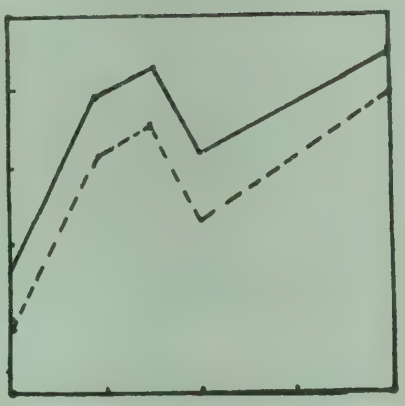
Carcinoma : resting juice of foul odour ; blood present ; no free HCl, high total (probably organic), lactic acid and mucus present.



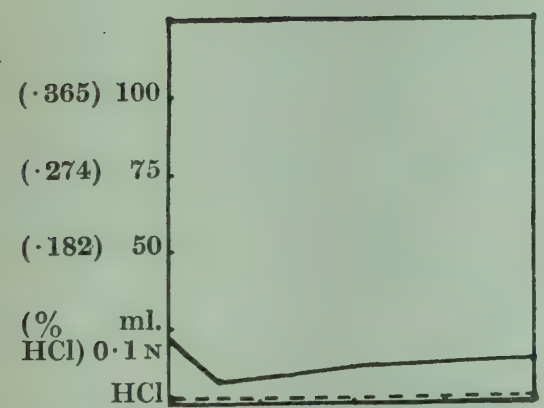
Pyloric ulcer : high free and total HCl ; no starch or mucus ; bile present ; no blood ; no lactic acid.



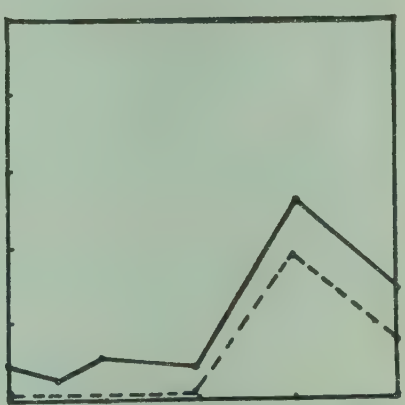
Duodenal ulcer; resting juice normal: marked response to stimuli in free and total HCl; slow emptying (plateau type); bile present, no blood.



Duodenal ulcer; free and total HCl rather high; no mucus, bile or blood.



Pernicious anaemia: no free HCl; low total; no starch; mucus and small amount of bile present.



Apparent achlorhydria (gastric ulcer); little mucus and bile; no blood.

FIG. 13.

Chapter XIII

VOLUMETRIC SOLUTIONS

A "normal" solution of any compound contains the equivalent of 1 gram of hydrogen (capable of reaction) in 1 litre of solution. Acids furnish the reactable hydrogen directly; they are said to have "replaceable" hydrogen. But in many cases, the relation of the substance to hydrogen is only obtained through an intermediate series of reactions.

NORMAL SULPHURIC ACID

Sulphuric acid ($H_2SO_4 = 98$) clearly contains 2 g. of hydrogen per gram mole, and hence $\frac{1}{2}$ gram mole ($= 98/2 = 49$ g.) of sulphuric acid is present in one litre of N-sulphuric acid. In practice, 27 ml. of concentrated acid (sp. gr. 1·84; hence 27 ml. approx. $\equiv$ 49 g.) are measured into about 800 ml. of cold water in a litre volumetric flask. When the solution is cold it is made to the mark and well mixed. This approximately N-acid is then standardized against pure anhydrous sodium carbonate by the reaction—

$$Na_2CO_3 + H_2SO_4 \longrightarrow Na_2SO_4 + CO_2 + H_2O$$

From this equation, 106 g. of sodium carbonate is equivalent to 2 l. of N-sulphuric acid, or 1·06 g. sodium carbonate $\equiv$ 20 ml. of N-sulphuric acid. About 1 g. of anhydrous sodium carbonate is therefore accurately weighed out on to a watch glass, and washed into a 100 ml. flask or beaker. To this solution, a few drops of methyl orange indicator are added, and the approximately N-sulphuric acid is run in from a burette until the indicator changes from yellow to orange (it is orange in acid solution, yellow in alkaline). The experiment is repeated.

From the above equation, the number of ml. of the approximately N-sulphuric acid which would be equivalent to 1·06 g. of sodium carbonate is calculated. This should be

less than 20 ml. Suppose, for example, it is 18·45 ml.; then every 18·45 ml. of the acid is equivalent to 20 ml. of N-acid. That is, 18·45 ml. of acid must be diluted with water to 20 ml., to give N-acid. An appropriate dilution of part or all of the remaining approximately N-acid is now made.

The mixed solution should be exactly N, and may be again tested against sodium carbonate to confirm this.

NORMAL SODIUM HYDROXIDE

Sodium hydroxide (NaOH = 40) contains in 40 g. of substance an amount of sodium (23 g.) which is equivalent to 1 g. of hydrogen. This "equivalence" of sodium to hydrogen is best understood by reference to the equation—

$$HCl + NaOH = NaCl + H_2O.$$

Approximately 41 g. of the solid are weighed out as quickly as possible, and put into 50 ml. of water in a litre flask. When cold, the solution is mixed and made to volume. This approximately N-solution is standardized against pure oxalic acid.

$$2NaOH + \begin{matrix}COOH\\|\\COOH\end{matrix} \longrightarrow \begin{matrix}COONa\\|\\COONa\end{matrix} + 2H_2O$$

90

Hence, 2 litres of N-sodium hydroxide are equivalent to 90 g. of anhydrous oxalic acid, or 90 + 36 = 126 g. of oxalic acid crystals, $\begin{matrix}COOH\\|\\COOH\end{matrix}.2H_2O$

Thus,

1·26 g. of oxalic acid crystals
≡ 20 ml. of N-sodium hydroxide.

Therefore about 1·2 g. of oxalic acid are accurately weighed out, washed into a flask, and the solution titrated with the approximately N-sodium hydroxide, with methyl red

(yellow in alkaline, red in acid solution) as indicator. The amount of the alkali equivalent to 1·26 g. of oxalic acid is calculated. This amount should be less than 20 ml. The dilution to exactly N is then accomplished, as in the case of sulphuric acid.

NORMAL AMMONIA SOLUTION

Concentrated (sp. gr. 0·88) ammonia solution is approximately 17 N. Fifty-eight ml. of this are therefore diluted with water to 1 litre, and the solution standardized against the N-sulphuric acid, with methyl red as indicator.

TENTH NORMAL POTASSIUM PERMANGANATE

Potassium permanganate ($KMnO_4$) reacts in acid solution with oxalic acid as follows :

$$\underset{2 \times 158}{2KMnO_4} + 5 \underset{\substack{\text{COOH} \\ 90 \\ (+\,2H_2O \\ =\,126)}}{\begin{vmatrix} \text{COOH} \\ \\ \text{COOH} \end{vmatrix}} + 3H_2SO_4 \longrightarrow K_2SO_4 + 2MnSO_4 + \underset{10CO_2 + 8H_2O}{}$$

1 gram mole of potassium permanganate is equivalent to 5 atoms of hydrogen. Hence, 0·1 N-permanganate contains $\frac{158}{50} = 3{\cdot}16$ g. per litre of solution. 3·16 g. of pure potassium permanganate are therefore weighed out, dissolved in water, and the volume made to 1 litre.

From the above equation, 5 × 126 g. of oxalic acid crystals ≡ 10 l. of N, ≡ 100 l. of 0·1 N-permanganate. That is, 0·126 g. of oxalic acid crystals ≡ 20 ml. of 0·1 N-permanganate. The above 0·1 N-solution may therefore be checked by titrating with it an exactly weighed amount (about 0·126 g.) of pure oxalic acid crystals, dissolved in approximately N-sulphuric acid. The acid solution is held in a beaker of hot water (about 80° C.) during titration, which is continued until the first persistence of pink permanganate colour.

TENTH NORMAL SODIUM THIOSULPHATE

Sodium thiosulphate ($Na_2S_2O_3$) reacts with iodine as follows :

$$2Na_2S_2O_3 + I_2 \longrightarrow 2NaI + Na_2S_4O_6$$

Hence, 1 gram mole of thiosulphate ≡ 1 gram atom of iodine and (since iodine reacts with hydrogen to give hydriodic acid, HI) 1 gram mole of thiosulphate is contained in 1 litre of N-solution. Approximately 0·1 N-sodium thiosulphate is made by dissolving 25 g. of the crystals ($Na_2S_2O_3.5H_2O$ = 248·2) in water, and making to 1 litre.* This solution is then standardized against a known amount of iodine, obtained from potassium biiodate ($KIO_3 . HIO_3$) solution by the reaction—

$$\underset{389\cdot97}{KIO_3.HIO_3} + 10KI + 11HCl \longrightarrow 11KCl + 6I_2 + 6H_2O$$

0·1 N-potassium biiodate is therefore made by dissolving $\frac{389\cdot97}{10\times12}$ = 3·250 g. of pure potassium biiodate in water in a litre flask, and making to volume. The titration is carried out as follows :—

A flask containing a solution of approximately 1 g. of potassium iodide in a little water is treated with about 10 ml. of approximately 5 N-hydrochloric acid (made by 50 per cent. dilution with water of the concentrated acid). Twenty-five ml. of the 0·1 N-potassium biiodate solution are then run in from a pipette. The iodine set free is titrated with the approximately 0·1 N-sodium thiosulphate, until a light yellow colour is obtained. Two drops of starch indicator are added, and the titration continued until the solution is colourless. The titration should be less than 25 ml. Dilution of the thiosulphate to exactly 0·1 N is carried out as in the previous cases.

Starch Indicator.—One hundred ml. of water are heated in a beaker to boiling. A paste of approximately 1 g. of starch in a little cold water is poured into the beaker. A few crystals of phenol red are added, and boiling is continued for a few minutes. The solution is cooled and preserved with a little chloroform.

* 1 ml. of 20 per cent. sodium carbonate and 10 ml. of amyl alcohol per l. may be included in the solution as a preservative.

TABLE 7.—TABLE OF NORMALITIES

Sp. Gr. at 15° C.	Normality	ml. conc. per litre to give normality
	Sulphuric Acid	
1·016	0·5	13·9
1·032	1·0	27·8
1·063	2·0	55·5
1·153	5·0	138·9
1·290	10·0	277·7
1·840	35·9	—
	Hydrochloric Acid	
1·008	0·5	50
1·017	1·0	100
1·034	2·0	200
1·083	5·0	500
1·16	10·0	—
	Nitric Acid	
1·017	0·5	31·7
1·033	1·0	63·4
1·067	2·0	126·7
1·165	5·0	316·8
1·305	10·0	633·6
1·420	15·75	—
	Acetic Acid	
1·0037	0·5	30
1·0083	1·0	60
1·0171	2·0	120
1·0412	5·0	300
1·0685	10·0	600
1·0553	16·16	—
	Ammonium Hydroxide	
0·9963	0·5	28·6
0·9925	1·0	57
0·9854	2·0	114
0·9620	5·0	286
0·9313	10·0	571
·9000	15·0	872
0·882	17·2	—

TENTH NORMAL IODINE SOLUTION

Approximately 13·5 g. of pure sublimed iodine are dissolved in a solution of 24 g. potassium iodide in about 200 ml. of water in a litre volumetric flask. The solution is diluted to the mark, mixed, and standardized against 0·1 N-sodium thiosulphate.

INDICATOR SOLUTIONS

Methyl Orange.—0·1 g. of methyl orange is dissolved in 100 ml. of water.

Methyl Red.—0·2 g. of methyl red in 100 ml. of alcohol.

Phenolphthalein.—0·5 g. of phenolphthalein is dissolved in 50 ml. of alcohol and 50 ml. of water are added.

Chapter XIV

COLORIMETRIC AND PHOTOMETRIC MEASUREMENTS

PHOTOMETRIC MEASUREMENT WITH THE ORDINARY COLORIMETER

Photometric measurement of the intensity of colour in a solution is preferable, for analytical purposes, to colorimetric comparison with standard solutions. More accurate measurement of the colour is possible, and interference by extraneous colours—a source of frequent trouble in colorimetry—can be avoided. Photometers are, however, expensive, and most laboratories are already equipped with colorimeters of the Duboscq type. By the use of light filters and neutral grey screens it is possible to make photometric measurements with the ordinary Duboscq colorimeter.* By placing the light filter on the top of the eye-piece, and using daylight or artificial illumination, virtually monochromatic light is obtained. The light source is then adjusted to yield exactly equal illumination on both sides of the field. Neutral grey screens serve as standards of light absorption.† The neutral screen is placed on the left-hand rack of the colorimeter, and the rack screwed up till the screen is against the bottom of the plunger. The coloured solution is placed in the right-hand cup and its depth is adjusted until the two fields appear equal. This depth gives the measure of the light absorbed by the solution, which is equal to that absorbed by the neutral grey screen. The absorption will vary for lights of different wave-length as given by the different filters. With the filter showing maximum absorption the reading

* The spectral filters of Messrs. Ilford Ltd. are appropriate for the purpose. A set of 8 gelatin spectral filters, mounted in glass (grade "A" glass, ¾ in. diameter, is suitable), can be obtained covering the visible spectrum.

† For example, Ilford neutral grey screens of 0·25, 0·50, and 0·75 densities. For most purposes the following set is sufficient: Ilford 41/45 violet, 807 green, 205 red, and 0·5 D grey ("A" glass, 1 in. diam.).

(millimeters of solution) will be minimum; and the depths of two different solutions of the same coloured substance should be in inverse ratio to the strengths of the solutions. Generally speaking, absorption will be found to be maximum for red solutions in the green or blue-green, and conversely, green solutions will show maximum absorption in the red. Blue and violet solutions are maximally absorbing in the yellow, orange, and red; and yellow and orange solutions in the blue and violet.

The grey screen of an appropriate density, together with the light filter showing maximum absorption, may be used as a permanent standard for any colorimetric method. It should be calibrated against the coloured solution of known strength — the "standard"; and the general equation for calculating the result for an unknown solution—the "test" —then becomes :—

$$\frac{\text{Read. of standard against grey screen}}{\text{Read. of test against grey screen}} \times \text{Conc. of standard} = \text{Conc. of test.}$$

The colour of any solution may also be expressed as its "extinction coefficient," (E). The calculation is as follows :

$$E = \frac{\text{Density of grey screen (e.g., 0·50)}}{\text{Reading of solution against grey screen (in cm.)}}$$

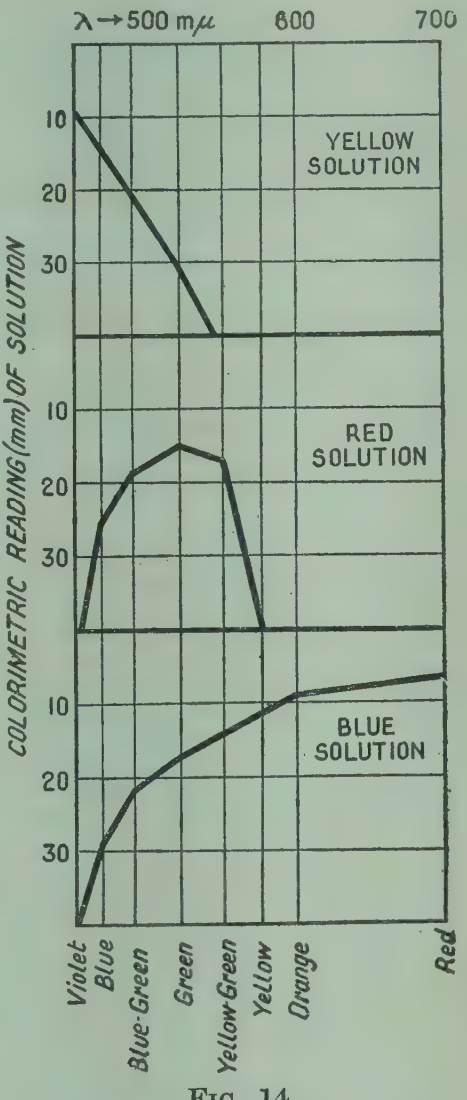


FIG. 14.

Absorption curves of standard solutions (neutral grey screens, spectral filters). Yellow solution: nesslerized ammonium chloride solution (strong urea standard); neutral screen, density 0·75. Red solution: bilirubin standard, 0·1 mg. in 25 ml. (Haslewood and King, 1937); neutral screen, density 0·50. Blue solution: uric acid standard; neutral screen, density 0·50.

When comparison of an unknown solution with a known standard of the same substance is made, using the light filter showing maximum absorption, the accuracy of matching is increased ; and interference by other contaminating colours is minimised because they are not maximally absorbing for light of the wave-length being used.

NOTE.—A fairly powerful light source is required with these filters. If the colorimeter is equipped with an artificial light it may be advantageous to increase the illumination by substitution of a stronger light bulb for that furnished.

DIRECT-READING PHOTOELECTRIC COLORIMETER

Duboscq colorimeters and photometers are now giving place to photoelectric colorimeters, which estimate the intensity of colour in a solution by measuring photoelectrically the proportion of incident light which is absorbed by the coloured solution. Typical instruments are the Evelyn (1936), the Hilger (1936), and the Summerson (1939). When light falls on a photoelectric cell the current generated can be measured by connecting the cell to a galvanometer. The degree of deflection of the galvanometer needle depends on the amount of current generated, and this depends on the amount of light falling on the photoelectric cell. If a coloured solution is interposed between the source of light and the photoelectric cell some of the light is absorbed, less electricity is generated and a smaller deflection of the galvanometer takes place. A more strongly coloured solution will absorb more light, still less electricity will be generated, and the deflection will be still smaller. The degree of deflection of the galvanometer, therefore, can be used to give a measure of the intensity of colour in the solution, and hence of the concentration of the substance being analysed.

The instrument here described makes use of this simple principle. It has been in use for routine and research in these laboratories since 1940 and has largely displaced the visual comparison colorimeters formerly in use. Some of the advantages of photoelectric colorimetry over visual colorimetry are the much greater speed and ease of colour

measurement, and the elimination of eye fatigue and personal error.

CONSTRUCTION

The construction and assembly of the instrument are illustrated in fig. 15. An ordinary surgical head-lamp furnishes the source of light (E). The bulb is activated from an accumulator or constant voltage transformer. A steady, non-fluctuating light is obtained. Immediately in front of the lamp is mounted a block of wood (well-seasoned teak or oak 3 × 3 × 2 in.) which has been bored vertically to fit a $\frac{5}{8}$-in. test-tube (A) and a $\frac{1}{2}$ × 2-in. metal bolt (B) whose nut is countersunk in the block. The bolt, which should fit its hole tightly, is used to vary the size of the slot through which the light passes. The slot is made by boring a $\frac{1}{2}$-in. hole from front to back, (passing through the vertical holes) and then sawing up from the bottom of the block to make the slot $\frac{1}{2}$ in. wide by $1\frac{1}{2}$ in. high. At the front of the block a groove is made 1 in. wide and $\frac{1}{8}$ in. deep to carry a light filter (D). A similar groove $\frac{7}{8}$ in. wide is made at the back of the block to fit the photoelectric cell (C). The block is mounted on a base board of wood, and the $\frac{5}{8}$-in. hole is extended $\frac{1}{4}$ in. into this so that the end of the test-tube drops below the bottom of the slot.

The photoelectric cell is of the selenium type, 22 × 40 mm. The "**EEL**" electroselenium cell supplied by Messrs. Evans of Bishop's Stortford has proved satisfactory. A copper wire is fitted on the surface of the rear groove in suitable position to make contact with the exposed strip of selenium which lies near the edges of the front surface of the cell. The wire is connected to an ordinary electric terminal screwed into the back of the block. A lead from this front-surface terminal connects with the negative pole of the galvanometer. A second terminal carries a spring clip which holds the photoelectric cell in place and makes contact with its back surface. The back-surface terminal is connected to the positive pole of the galvanometer.

Light filters of glass have been used. The Chance red (OR 2), green (OGr 1), and blue-green (OB 2) glass filters have been found suitable for most purposes. Alternatively, Ilford

tricolour red, tricolour green, and minus red gelatin filters may be used. The most useful size is 1 × 2 in. Both the sensitivity and accuracy of colour measurement are increased by the use of light filters. As in visual photometry, the measurement of colour intensity by photoelectric means is best carried out in light restricted to parts of the spectrum whose light is maximally absorbed by the coloured solution under investigation. This condition is approximated with the filters recommended.

The galvanometer for use with this instrument should be of 500–1000 ohms resistance and should have a maximum deflection corresponding to 5–10 microamperes. The

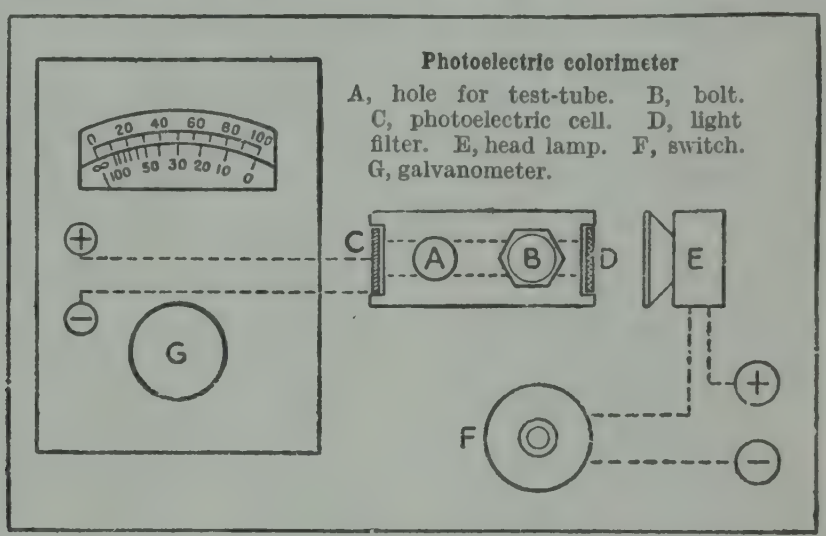


FIG. 15.—Direct-reading Photoelectric Colorimeter.

pointer type and the reflector type have been found equally satisfactory. The most convenient galvanometer scale is a logarithmic one on which the zero point corresponds to maximum deflection. The galvanometer reading on the logarithmic scale is then directly proportional to the light absorbed. A set of coloured solutions containing varying amounts of the same pigment will give readings which are directly proportional to the concentration.

The logarithmic scale used is, in fact, that of the extinction "E." If the full deflection of the galvanometer is divided into a linear scale of 100 equal parts, on which 0 corresponds to darkness and 100 to the point of full-scale deflection with 100 per cent. of incident light, then any readings less than

100 will correspond to percentages of light transmission which are less than 100 per cent. of the incident light. They can be thought of as different degrees of extinction of the incident light. The extinction E or " optical density " is defined as the logarithm of the ratio of incident to transmitted light—

$$\text{i.e., } E = \log. (100/\% \text{ transmitted light}).$$

If two solutions of different optical density are interposed in the light path, then their extinctions E will be in the same relation one to the other as their concentrations of pigment —i.e., $E_1/E_2 = C_1/C_2$. This simple photometric expression of Beer's law defines the condition to be aimed at with a photoelectric colorimeter. To simplify the reading of E, and hence to simplify the calculation, the logarithmic scale is used on the galvanometer. It is arrived at in the following way. If, for instance, a coloured solution transmits only 50 per cent. of the incident light—i.e., gives a reading of 50 on the linear scale—then its extinction $E = \log. 100/50 = 0 \cdot 301$. The point opposite 50 on the linear scale is marked $0 \cdot 301$ on the logarithmic scale. Similarly, 100 on the linear scale is 0 on the logarithmic, 10 on the linear is 1 ($\log. 100/10 = 1$) on the logarithmic, and so on. For convenience, the values of E, which comprise the logarithmic scale, are multiplied by 100, and are plotted as whole numbers. The extinction E of a coloured solution is spoken of as the " extinction coefficient " when it is determined at, or reduced to, a solution depth of unity—i.e., 1 cm.

OPERATION OF THE INSTRUMENT

The galvanometer is levelled, the suspension is released and the " spot " or pointer adjusted to the ∞ mark at the left of the logarithmic scale (0 on the linear scale) by turning the suspension knob. The light is now switched on. The bolt is turned up or down to regulate the amount of light reaching the photoelectric cell, until the needle is at 0. It should remain at this point and will do so after the first few minutes if the electric supply is taken from a properly charged accumulator or adequate transformer.

A set of test-tubes is selected which will fit the hole snugly but easily. They should be all of similar glass and free from obvious streaks and scratches. A test-tube is half filled with water, wiped with a towel to remove any stains and placed in the hole. The bolt is turned until the needle is at 0. A scratch is made on the side of the test-tube opposite a mark on the block to indicate that the tube should always be used in that position. If on turning the tube in the hole more than a scarcely perceptible movement of the needle takes place the tube should be discarded since it contains flaws in the glass which are interfering with the passage of light. The other tubes are tested similarly and a mark made on each to indicate in which position it should be inserted to ensure equal light transmission. These tubes may now be used interchangeably. The reactions leading to the production of the coloured solutions may be carried out in them, so that no transference of the solution is necessary when it is read in the colorimeter. A good quality of fairly thin-walled test-tubes should be used.* The most convenient size is $\frac{5}{8} \times 5$ in. It will be found useful to mark them with a file to indicate volumes of 5 ml., 10 ml. and 15 ml.

The appropriate light filter for a coloured solution is next tested for. This will usually be the filter which gives the highest reading—i.e., absorbs most light—and which gives most nearly a direct proportionality in reading with different concentrations of pigment. Generally speaking, its colour will be " complementary " to that of the solution—for a blue or green solution a red filter, for a yellow solution a blue filter, and for a red solution a green filter. The selection of the filter is made as follows :—

With the filter and test-tube of water in place, the galvanometer needle is adjusted to 0 by turning the bolt. The test-tube of water is removed and another containing the colour standard is inserted. The galvanometer needle is allowed to come to rest and the reading is recorded. On replacing the tube of water the needle should return to 0. If it fails to do so, the light is again adjusted by turning the bolt and the reading

* For more accurate work the very thin-walled and uniform test-tubes of the type used in the Lovibond comparator are useful.

TABLE 8.—ADAPTATIONS OF MICRO-CHEMICAL METHODS FOR USE WITH THE PHOTOELECTRIC COLORIMETER

Substance	Changes necessary	Light filter
Urea . .	Use a blank of 7 ml. H_2O + 1 ml. Nessler's for 0 adjustment of galvanometer	Chance OB. 2 or Ilford minus red.
Uric Acid .	Dilute tests and standard to 5 ml. with water (very high ones to 10)	Chance OR. 2 red, or Ilford tricolour red.
Creatinine .	Dilute tests and standard to 5 ml. with water. Use a blank consisting of water + 0·5 ml. of alkaline picrate	Chance OB. 2 or Ilford minus red.
Phosphate .	No change	Chance OR. 2 red, or Ilford tricolour red.
Cholesterol .	No change	Chance OR. 2 red or Ilford tricolour red.
Glucose . .	Colorimetric method. Dilute with water to 10 ml., or 15 or 20 ml. for very high sugars. Use a blank for 0 adjustment	Chance OR. 2 or Ilford tricolour red.
Sulphonamides	No change	Chance OGr. 1 green or Ilford tricolour green.
Proteins and N.P.N. .	Use a blank consisting of 0·2 ml. 50 % H_2SO_4, 5 ml. water and 3 ml. Nessler's	Chance OB. 2 or Ilford minus red.
Bilirubin . .	Use 7 ml. of 85 % alcohol instead of 3 ml. abs. alcohol. This gives final vol. of 8 ml. instead of 4 ml. and standard is now equal to 3·2 mg. bilirubin per 100 ml.	Chance OGr. 1 green or Ilford tricolour green.
Sodium . .	1 ml. of coloured solution diluted to 10 ml. with 0·5 % acetic acid	Chance OB. 2 or Ilford minus red.
Phosphatase .	Dilute to 10 ml. with water. (High phosphatase further diluted)	Chance OR. 2 red. or Ilford tricolour red.
Potassium .	No change	Chance OR. 2 red or Ilford tricolour red.
Haemoglobin .	All methods described	Chance OGr. 1 green or Ilford tricolour green.

for the standard is again taken. This operation is now repeated with a second standard of double the strength of the first. The reading of the second standard should be twice that of the first. If this condition is not fulfilled, another light filter is tried. If the proportionality is approximated but not reached, the fault probably lies in the presence of a "blank" colour—i.e., a colour arising from the reagents which have been used to produce the colour by reaction with the standard substance. A "blank" consisting of the reagents and water, with no added standard, is prepared and substituted for the water in the 0 adjustment. Proportionality should now be obtainable with the appropriate filter.

Use with Standards. The use of a natural standard—that is, a solution containing a known amount of the substance under investigation—is almost universal in colorimetric practice with Duboscq type instruments. The preparation of a standard each time a determination is made has another use besides furnishing a basis for colorimetric comparison—it is the easiest way of testing if anything has gone amiss in the analytical procedure. For this reason the use of standard solutions should be continued with the photoelectric colorimeter. When the present direct-reading type, with a logarithmic scale, is used the calculation is simply that of direct proportionality—

(Reading of test/Reading of standard) $\times$ Concentration of standard = Concentration of test.

The factor Reading of test/Reading of standard is reversed from that used with the Duboscq instrument, since the proportionality is here a direct, whereas with the Duboscq it is an inverse proportionality.

The micromethods of analysis for blood which have been described have been readily adjusted for use with the photoelectric instrument. Very few changes have been necessary: they are given in the table, together with the light filter recommended for each method. As in visual colorimetry the reading of the standard which is nearest the test should be used in the calculation.

Use without Standards. A coloured solution of given density will always absorb the same proportion of incident

light, provided the light is invariably of the same intensity and wave-length. With any given coloured solution and the proper light filter, therefore, the same difference in galvanometer deflection between water and coloured solution should be obtained. This is the case for any one instrument ; different instruments may differ sufficiently in details of construction to give slightly different deflections with the same solution. By calibrating an instrument against a series of standards a factor may be derived or a graph constructed for the calculation of the concentrations of subsequent test solutions of unknown strength. But it must be recognized that the accuracy obtained in this way may not be as great as that which will result from the simultaneous comparison of a standard with the test solution. The reagents used in any procedure may vary slightly from day to day—e.g., by deterioration—and as a consequence the colour produced on one occasion may not be absolutely identical with that produced on another. Moreover, the selenium cell may suffer small alterations in its sensitivity and as a consequence the deflection resulting from any given incident light may vary slightly. However, the accuracy obtainable may be sufficient for many routine examinations. If it is desired to use the colorimeter in this way, a separate set of standard graphs or factors should be established for each instrument.

PERMANENT STANDARDS FOR THE TURBIDOMETRIC ESTIMATION OF PROTEIN

The turbidometric procedure of Kingsbury *et al.* (1926) can be applied to urine, C.S.F. and other body fluids, and has been in use, with modifications in the apparatus required, in the laboratories of the British Postgraduate Medical School for some time. The procedure is very simple, and can be used with equal success by laboratory workers and general practitioners.

The principle of the method consists in comparing the turbidity in a set of permanent standards with that produced when a standard amount of sulphosalicylic acid is added to a measured amount of the albuminous fluid. The

standards consist of a permanent suspension of formazin in gelatin. They have been standardized in this laboratory by reference to the turbidity produced with sulphosalicylic acid in a solution of crystalline horse-serum albumin, a diluted human serum whose protein content was established by Kjeldahl, and a solution of crystalline albumin isolated from the urine of an orthostatic albuminuric.

The Method. Add 3 ml. of 3 per cent. sulphosalicylic acid to 1 ml. of urine, C.S.F., or other albuminous body fluid, in a small test-tube, and compare the turbidity after 5 minutes with that in the standards. If the degree of turbidity is too great for comparison make a suitable dilution of the fluid with water, and repeat the test.

Preparation of the Standards. The gelatin is clarified as follows : 65 g. of pure gelatin are dissolved at about 90° C. in 500 ml. of water. The " white " of one egg, in approximately twice its volume of water, is vigorously stirred into the solution. The mixture is heated, with continual stirring, on a boiling water-bath for 1 hour. It is then filtered through a large coarse paper in a heated funnel. The clear slightly yellow filtrate is kept liquid at about 50° C. for the preparation of the standards described below.

The formazin is prepared as follows : 25 ml. of an aqueous solution (10 g. per 100 ml.) of hexamine (hexamethylene tetramine) are added to 25 ml. of a solution (1 g. per 100 ml. in water) of hydrazine sulphate. The mixture is stoppered, shaken, and left at room temperature for at least 15 hours. The resulting precipitate of formazin is carefully mixed by gentle shaking until it is evenly dispersed throughout the liquid.

14·5 ml. of the suspension are added to 100 ml. of gelatin together with 0·3 ml. of 40 per cent. formaldehyde to ensure permanent " setting." Such a gelatin suspension of formazin has been found to be equivalent to a concentration of serum albumin (precipitated with sulphosalicylic acid under the conditions described above) of 100 mg. per 100 ml. The gelatin suspension is now diluted with clarified gelatin (containing 0·3 ml. of 40 per cent. formaldehyde per 100 ml.) to give standards corresponding to other albumin concen-

trations. The following mixtures of gelatin and formazin-gelatin suspension are made in small tubes of uniform bore (7·5 by 1 cm.)

TABLE 9

Tube	ml. of gelatin.	ml. of gelatin-formazin	Value in terms of albumin per 100 ml.
1	3·6	0·4	10
2	3·2	0·8	20
3	2·8	1·2	30
4	2·4	1·6	40
5	2·0	2·0	50
6	1·6	2·4	60
7	1·2	2·8	70
8	0·8	3·2	80
9	0·4	3·6	90
10	0	4·0	100 ·

When cold, the tubes are stoppered with corks cut level with the top of the tube. The stoppered ends are then dipped in molten paraffin wax and allowed to cool. The standards may be checked against serum solutions standardized by nitrogen determinations. They should be mounted in a wooden rack painted black. Comparison is best made by viewing them against a strip of white cardboard with a transverse black line fastened to the rack.

Arrangements have been made with Messrs. Gallenkamp and Co., London, to manufacture these standards. Sample sets have been checked against those prepared in this laboratory and against albumin solutions of known strength.

COLORIMETRIC STANDARDS FOR EMERGENCY ESTIMATIONS OF CERTAIN CONSTITUENTS OF BLOOD AND C.S.F.

The determination of urea in blood is most easily carried out by conversion of the urea into ammonia by urease, followed by direct Nesslerization; that of non-protein nitrogen (N.P.N.) and plasma proteins by suitable digestion with sulphuric acid, whereby the whole of the contained

nitrogen is converted into ammonia, followed by Nesslerization. The resulting yellowish-brown solution is compared with a suitable standard in a Duboscq or photoelectric colorimeter, or in a photometer. Such instruments are not always available to the clinician or to the pathologist working under emergency conditions.

With the purpose of providing a simple means of estimating these substances without the use of elaborate apparatus, artificial coloured standards for comparison have been devised. Such standards should be indistinguishable in day or artificial light from the "Nessler-yellow" colour and should have a fair degree of permanency. Of the various substances tried a mixture of ferric chloride and cobalt chloride in hydrochloric acid solution has been the most satisfactory.

In the standards described the proportions and amount of ferric chloride and cobalt chloride have been so adjusted that the resultant mixtures have appeared indistinguishable from Nesslerized ammonia solutions in daylight and in artificial light. The comparisons have been made in test-tubes of standard bore ($5 \times \frac{5}{8}$ in.) viewed in a comparator block against frosted glass and in a test-tube rack against white paper. A standard ammonium chloride solution containing the equivalent of 0·01 mg. of N per ml. was employed as a basis for the comparisons. This solution was used in varying amount with added water and 1 ml. of Nessler's reagent in a total volume of 8 ml. This is the final volume of test and standard solutions in the micro-methods for blood urea, N.P.N., and plasma protein which are used with the permanent iron-cobalt standards. The numbers shown in the first column of Table 10 refer to the amount of nitrogen (mg. N) present as Nesslerized ammonia in the volume of 8 ml. which is the equivalent in colour of the stated quantities of iron chloride and cobalt chloride contained in the same volume.

The standard values have been chosen to represent the concentrations most often encountered clinically. They are so spaced as to represent increments of 0·005 or 0·01 mg. N, corresponding to 10 or 20 mg. of N.P.N. or urea per 100 ml. of blood. In the lower and middle range the

TABLE 10.—COMPOSITION OF PERMANENT STANDARDS

Standard value (N equivalent) mg.	ml. of Ferric Chloride Solution *	ml. of Cobalt Chloride Solution *	0·1 N-HCl.
0·015	1·1	0·15	0·1 N-HCl added to make the volume 8 ml.
0·02	1·4	0·27	
0·025	1·75	0·40	
0·03	2·1	0·55	
0·035	2·4	0·68	
0·04	2·75	0·80	
0·045	3·1	0·93	
0·05	3·4	1·05	
0·055	3·75	1·18	
0·06	4·1	1·30	
0·07	4·7	1·55	
0·08	5·4	1·80	
0·09	5·9	2·10	

* Ferric chloride solution: 10 per cent. (w./v.) $FeCl_3.6H_2O$ in 0·1 N-hydrochloric acid—i.e., 10 g. of the salt dissolved in enough 0·1 N-hydrochloric acid to make a final volume of 100 ml. Cobalt chloride solution: 10 per cent. (w./v.) $CoCl_2.6H_2O$ in 0·1 N-hydrochloric acid.

values are spaced more closely in order to allow of more accurate estimation of plasma proteins. For total proteins the increments of 0·005 mg. N correspond to 0·78 per cent. protein. By careful matching intermediate values can be assigned, and it should hence be possible to gauge the concentration of protein to within 0·4 per cent. On the same basis it should be possible to measure fibrin to within 0·02 per cent. and albumin to within 0·2 per cent. Since globulin is estimated by the difference between total protein and albumin the error may be additive, and at the most should be 0·6 per cent. For C.S.F. protein the increments of 0·005 mg. N correspond to 6 mg. per 100 ml. of protein, and half-increments to 3 mg.

An alternative rapid method for plasma and C.S.F. proteins, based on the xanthoproteic reaction, may also be used.

The colour of iodine in ether solution is very much like that of the Nessler-yellow. The estimation of sugar by a method in which the final determination is of liberated iodine should make it possible to estimate blood sugar by means of the permanent standards. The Schaffer-Hartman procedure was easily adapted to this purpose. The intensity of the

iodine colour is in inverse proportion to the amount of sugar : the more sugar there is present the more iodine will be used to reoxidize cuprous copper ; therefore, the lower values in the permanent standards correspond to high sugar values, and the higher standards to low sugar values. The figures for blood sugar obtained in this way are, of course, only rough estimates of the true values, but they are close enough to be of clinical use. In practice it has been found that figures within 10 or 15 mg. of the known values of dilute sugar solutions or the determined values (by an accurate method) of blood filtrates can be obtained.

METHODS OF ANALYSIS FOR USE WITH PERMANENT STANDARDS

Blood Urea (p. 5). The colour is matched with the standards and the figure obtained (mg. N) multiplied by 2,140—i.e., $\frac{100}{0 \cdot 1} \times 2 \cdot 14$—to give the mg. urea per 100 ml. *C.S.F. Urea* may be estimated in the same way.

Non-protein Nitrogen (p. 7). The figure obtained on matching with the standards is multiplied by 2,000—i.e., $\frac{100}{0 \cdot 05}$—to give the mg. N.P.N. per 100 ml. of blood.

Plasma Total Protein : (1) *By Difference with N.P.N.* (p. 35). The value (mg. N) is multiplied by 20,000—i.e., $\frac{100}{0 \cdot 005}$—to obtain the total N per 100 ml. The N.P.N. value (as mg. per 100 ml.) is found for the plasma or serum, and is subtracted from the total N per 100 ml.* The resulting figure is divided by 1,000 and multiplied by 6·25 to obtain the number of grams of total protein per 100 ml.—i.e., the percentage of protein.

(2) *By Precipitation of the Protein* (p. 34). The figure

* If the mg. of blood urea per 100 ml. is already known and be not grossly elevated it will be sufficiently accurate to subtract this figure from the total N instead of performing the plasma N.P.N. estimation. In cases where there is no reason to suspect nitrogen retention the normal average N.P.N. figure for plasma—namely, 25 mg.—may be used.

(mg. N) obtained on comparing with the standards is multiplied by 125—i.e., $\frac{100}{0{\cdot}005} \times \frac{6{\cdot}25}{1000}$ —to obtain the percentage of total protein.

Albumin (p. 36). The following are the calculations entailed :

(1) *By Difference with N.P.N.* :

$$\text{mg. albumin N} + \text{N.P.N.} = \text{Reading} \times 10{,}000 \left(\text{i.e., } \frac{100}{0{\cdot}01}\right)$$

$$\text{mg. albumin N} = (\text{Reading} \times 10{,}000) - \text{N.P.N.}$$

$$\text{percentage albumin} = \frac{\text{mg. albumin N} \times 6{\cdot}25}{1000}$$

(2) *By Precipitation of the Albumin* :

$$\text{percentage albumin} = \text{Reading} \times 62{\cdot}5 \left(\text{i.e., } \frac{100}{0{\cdot}01} \times \frac{6{\cdot}25}{1000}\right)$$

Globulin. Total protein minus albumin.

Fibrin. The value obtained for the colour on matching with the standards is multiplied by 6·25—i.e., $\frac{100}{0{\cdot}1} \times \frac{6{\cdot}25}{1000}$; the result is the percentage of fibrin.

C.S.F. Protein. 0·1 ml. of C.S.F. is digested with 0·2 ml. of 50 per cent. sulphuric acid as in the method for plasma total protein. The value obtained on comparing with the standards is multiplied by 1000—i.e., $\frac{100}{0{\cdot}1}$ —to give the total mg. N per 100 ml. C.S.F. The N.P.N. of the C.S.F. is estimated by digestion and Nesslerization of 1 ml. of a 1 : 10 protein-free filtrate, equivalent to 0·1 ml. C.S.F. (0·5 ml. of C.S.F. + 3·5 ml. of water + 1 ml. of 25 per cent. trichloroacetic acid). The result is multiplied by 1,000 to give the N.P.N. (mg. per 100 ml. of C.S.F.). The N.P.N. is subtracted from the total N to give the mg. protein N in 100 ml. This figure is multiplied by 6·25 to give the mg. protein per 100 ml. C.S.F.

Estimation of Plasma and C.S.F. Protein by the Xanthoproteic Reaction. When protein solutions are heated with

nitric acid a yellow colour is produced. This is intensified by the subsequent addition of excess of alkali. The colour is very similar to that of the permanent standards. Its intensity increases with the amount of protein present, and the relation between colour and amount of protein is almost a straight line. The test is very easy to carry out and constitutes a simple method of estimating protein in solution. The results are in good agreement with those obtained by the H_2SO_4-digestion and Nesslerization procedure. One ml. of C.S.F. or of 1 in 20 diluted plasma (for example, 0·5 ml. plasma diluted to 10 ml. with isotonic chloride or sulphate) is treated with 1 ml. of concentrated nitric acid. The mixture is heated rapidly to the boil, cooled immediately, and 2 ml. of sodium hydroxide (40 per cent.) added. The colour produced is compared with the standards and the protein content derived from Table 11.

TABLE 11.—PROTEIN EQUIVALENTS OF COLORIMETRIC STANDARDS BY XANTHOPROTEIC REACTION

Standards	mg. Protein per 100 ml. C.S.F.	Per cent. Protein in Plasma	Standards	mg. Protein per 100 ml. C.S.F.	Per cent. Protein in Plasma
0·015	50		0·055	280	5·6
0·020	70		0·06	310	6·2
0·025	95		0·065	340	6·8
0·03	120	2·3	0·07	370	7·4
0·035	150	3·0	0·075	400	8·0
0·04	185	3·7	0·08	430	8·6
0·045	220	4·4	0·09	500	10·0
0·05	250	5·0			

Sugar. 0·2 ml. of blood is diluted with 3·2 ml. of isotonic sodium sulphate and the proteins precipitated by 0·3 ml. of 10 per cent. (w./v.) sodium tungstate and of 7 per cent. (w./v.) copper sulphate ($CuSO_4.5H_2O$). The mixture is centrifuged or filtered, and 2 ml. of filtrate are treated with 0·3 ml. of Harding's copper reagent in a standard-bore test-tube. The tube is heated in a boiling water-bath for 10 minutes. It is then cooled and 0·5 ml. each of 1 per cent. potassium iodide

and of normal H_2SO_4 are added. Two ml. of ether * are now added and the mixture well shaken. The iodine will be seen to have gone into the ether layer. The colour is compared with the standards, and the sugar value derived from Table 12.

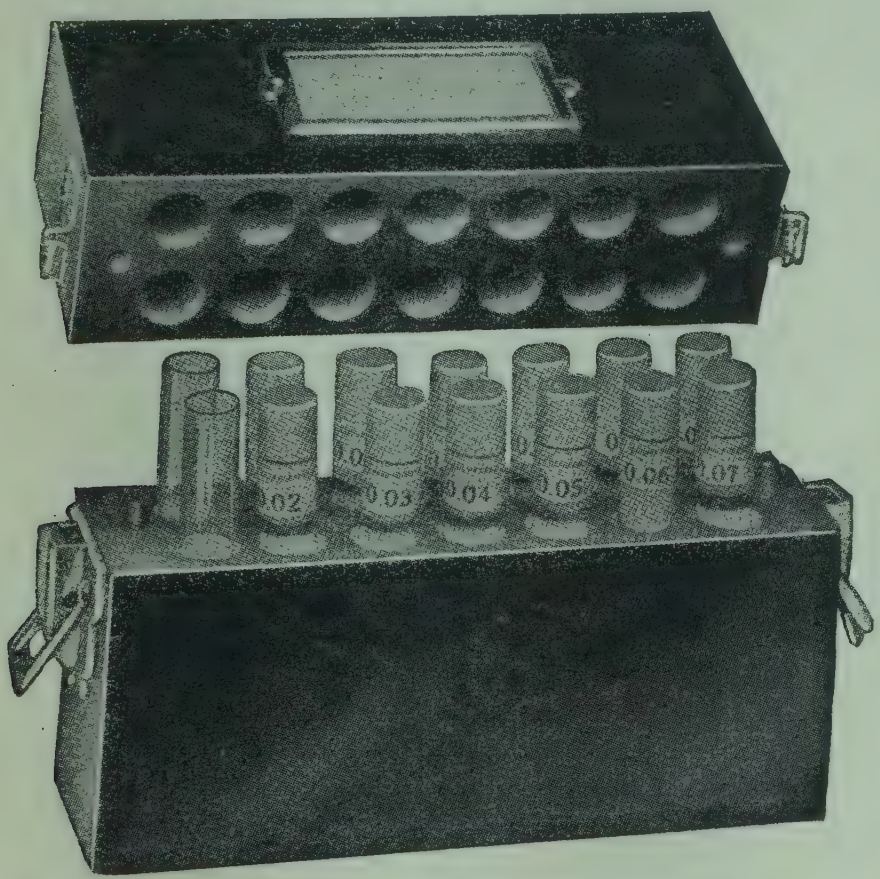


FIG. 16.—Permanent artificial colour standards for the emergency estimation of blood and C.S.F. constituents.

If the colour obtained is less than the 0·02 standard the test is repeated with 1 ml. of blood filtrate plus 1 ml. of water, and the result multiplied by 2. *C.S.F. sugar* may be estimated by the same procedure.

* It is essential that the ether be peroxide-free; otherwise excess iodine may be liberated from the potassium iodide. The ether may be tested by adding 2 ml. to a mixture of 2 ml. of water, 0·5 ml. of potassium iodide, and 0·5 ml. of sulphuric acid. If on shaking the mixture no perceptible yellow colour is present in the upper layer the ether is satisfactory for the test. In practice only a few samples of ether which had been stored in glass for a long time have proved unsatisfactory.

TABLE 12.—BLOOD-SUGAR EQUIVALENTS OF COLORIMETRIC STANDARDS

Standards	mg. Glucose per 100 ml.	Standards	mg. Glucose per 100 ml.
0·02	170	0·055	115
0·025	165	0·06	105
0·03	160	0·065	95
0·035	155	0·07	85
0·04	145	0·08	65
0·045	135	0·09	50
0·05	125		

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